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BACTERIOSTATIC EFFECT OF ANIONIC AND NONIONIC SURFACE ACTIVE AGENTS ON MYCOBACTERIUM TUBERCULOSIS H37Rv[1]

By HISAKO NISHIHARA

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(Received for publication, May 14, 1954)

As part of our studies on the effect of surface active agents on the tubercle bacilli, representative products of cationic, anionic, and nonionic types were selected from some four hundred samples submitted to us, and tested for their bacteriostatic effects. The results of the studies with the anionic and nonionic surface active agents are presented here.

EXPERIMENTAL

As previously described (*1*), 0.1 ml. of 1 mg. per ml. suspension of *Mycobacterium tuberculosis* H37Rv[2] was inoculated into vials containing the solutions of the test substances in Yomans' modification of the Proskauer-Beck liquid synthetic medium. The vials were incubated, and observed weekly for growth for 6 weeks.

RESULTS

The results are tabulated (Tables I to VII) as inhibition indices (I.I.), which were determined as previously described (*1*). An index of 10 indicates complete growth inhibition; O, no inhibition; and intermediate values, partial inhibition.

DISCUSSION

None of the anionic and nonionic surface active agents was completely bacteriostatic at $1:10^6$ dilution. All but two were completely bacteriostatic at $1:10^4$, and some, at $1:10^5$. In general these products were less effective than the cationic agents, most of which were completely inhibitory at $1:10^5$ (*1*, *2*).

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[1] This work was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

[2] Kindly provided by William Steenken, Jr., The Trudeau Foundation, Trudeau, New York.

TABLE I

Bacteriostatic Effect of Aninonic Surface Active Agents on Mycobacterium tuberculosis H37Rv

Salts of Phosphoric Acid Esters and Amides

Surface active product3)	Dilution4)	pH	S.T.	I.I.
1. $Na_5R_5(P_3O_{10})_2$ 70% paste	1 : 10^4	6.7	39	10
R=2-ethylhexyl	1 : 10^5	6.8	53	0
(Victawet 35B, 17)	1 : 10^6	6.9	61	0
2. $Na_5R_5(P_3O_{10})_2$ 70% paste	1 : 10^4	6.9	28	10
R=capryl	1 : 10^5	6.9	49	0
(Victawet 58B, 17)	1 : 10^6	6.9	54	0
3. Octadecylamine salt of esterified octadecyl-	1 : 10^4	6.8	32	10
amidophosphoric acid	1 : 10^5	7.0	53	7
(Victamine D, 17)	1 : 10^6	7.1	54	2
4. Octadecylamine salt of ethyloctadecyl-	1 : 10^4	7.0	35	10
amidophosphate	1 : 10^5	7.1	46	6
(E-2546, 17)	1 : 10^6	7.3	59	0

TABLE II

Bacteriostatic Effect of Anionic Surface Active Agents on Mycobacterium tuberculosis H37Rv

Alkylaryl Sulfonates

Surface active product3)	Dilution4)	pH	S.T.	I.I.
5. Sodium alkylbenzenesulfonate	1 : 10^4	7.0	40	10
40%, 60% sodium salts	1 : 10^5	6.9	53	0
(*, 19)	1 : 10^6	7.0	58	0
6. Alkylarylsulfonate 100%	1 : 10^4	6.9	32	10
(Santomerse 3, 12)	1 : 10^5	6.9	45	10
	1 : 10^6	7.0	62	0
7. Sodium xylenesulfonate 40% aqueous solution	1 : 10^4	6.9	64	0
(Ditto, 19)	1 : 10^5	6.9	66	0
	1 : 10^6	6.9	65	0
8. Decylbenzenesulfonate 100%	1 : 10^4	7.0	32	10
(Santomerse D, 12)	1 : 10^5	7.0	44	10
	1 : 10^6	6.9	61	0
9. Sodium alkylnaphthalene sulfonate 30% sol.,	1 : $10^{4.5}$	6.9	52	10
10% sodium sulfate (Wetsit WP, 18)	1 : $10^{5.5}$	6.8	57	0
	1 : $10^{6.5}$	6.8	61	0
10. Heptadecylbenzimidazoldisulfonate 100%	1 : 10^4	7.2	57	10
(Ultravon W, 6)	1 : 10^5	7.2	51	2
	1 : 10^6	7.1	45	0
11. Polymerized sodium salt of substituted	1 : 10^4	6.8	60	0
benzoid alkylsulfonic acids 85%	1 : 10^5	7.9	60	0
(Daxad 23, 7)	1 : 10^6	7.0	62	0

TABLE III

Development of Hydrogen Sulfide, Carbon Dioxide and Hydrogen from Lactate, Pyruvate and Formate

Expt. No.	Hydrogen donor		Acceptor	Gas formed		
				H_2S	CO_2	H_2
1	Lactate	3 μM	Na_2SO_4	0.6 μM	3.0 μM	0 μM
	None		//	0.2	1.7	0
		diff.		0.6	1.3	0
2	Pyruvate	4.9 μM	Na_2SO_3	0.6	2.7	0.1
	None		//	0.1	0.6	0
		diff.		0.5	2.1	0.1
3	Lactate		None	0.4	2.4	0.2
	None		//	0.1	0.6	0.3
		diff.		0.3	1.8	-0.1
4	Pyruvate		None	-0.2	3.7	2.2
	None		//	0.2	0.8	0.2
		diff.		-0.4	2.9	2.0
5	Formate		None	-0.1	3.8	1.9
	None		//	0	1.1	-0.2
		diff.		-0.1	2.7	2.1

Expt. Nos. 1 and 2: Each vessel contains suspension in $M/15$ phosphate buffer (pH 7.0) 1.5 ml., $M/10$ Na_2SO_4 0.5 ml. in main chamber, hydrogen donors or water 0.2 ml. in side arm I, H_2SO_4 diluted 0.3 ml. in side arm II, and absorption solution 0.3 ml. in center well. Temperature: 37°. Gas phase: nitrogen.

Expt. Nos. 3, 4 and 5: Each vessel contains cell suspension 3 ml. in main chamber, $M/10$ hydrogen donors or water 0.2 ml. in side arm I, H_2SO_4 diluted in side arm II and absorption solution 0.3 in center well. The reaction time: 1 hour. Temperature: 37°. Gas phase: nitrogen.

DISCUSSION

It was found that our strains of sulfate-reducing bacteria reduce sulfate, sulfite and thiosulfate almost quantitatively to hydrogen sulfide. This observation proved that sulfate, sulfide and thiosulfate could be used as hydrogen acceptors by sulfate-reducing bacteria.

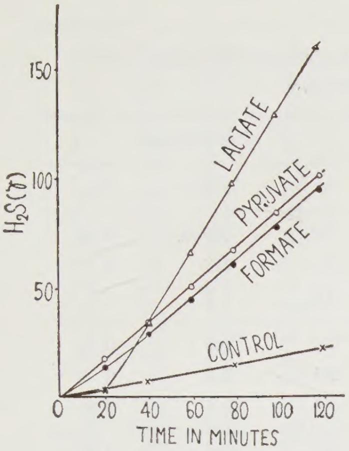

Fig. 1. Development of hydrogen sulfide from sulfate by lactate, pyruvate and formate.

Condition of reaction mixtures contained cell suspension 3 ml. in *M*/15 phosphate buffer, pH 7.0, *M*/10 Na_2SO_4 1 ml. and *M*/10 substrate 1 ml. Temperature: 37°.

(4) $2CH_3CHOHCOOH+H_2SO_4=2CH_3 \cdot COOH+H_2S+2H_2O+2CO_2$

(5) $4CH_3COCOOH+H_2SO_4=4CH_3 \cdot COOH+H_2S+4CO_2$

(6) $4HCOOH+H_2SO_4=H_2S+4CO_2+4H_2O$

As the above table shows, the observed values were approximate to the theoretical values. Therefore it was concluded that the oxidation of lactate, pyruvate and formate may be caried out according to Eqs. (4), (5) and (6).

At the same time we found that our strains decomposed pyruvate and formate in the absence of hydrogen acceptors such as sulfate. Hydrogen, but not hydrogen sulfide, was evolved. The rates of the evolution of hydrogen in such a case were dependent on the concentration of these compounds. When the concentration was low the rates were small. The ratios of hydrogen gas to carbon dioxide evolved from pyruvate or formate were almost 1 :1, as shown in Table III. Therefore it was clearly established that this strain posessed hydrogenlyase and formate was decomposed by this enzyme. From lactate even in the absence of sulfate there was no appreciable hydrogen evolution, but carbon dixide developed slightly. It may be due to the presence of trace of hydrogen acceptors and not to the dismutation of lactate.

Effects of Inhibitors

We made many experiments on the effects of various inhibitors on the reduction of sulfate, sulfite, thiosulfate and dyes. The results are listed in Table IV.

The reduction of sulfate with lactate or molecular hydrogen was strongly inhibited by cyanide, selenate and arsenite at the low concentration. But these inhibitors did not affect the reduction of dyes at the same low concentration. The reduction of sulfite also was more sensitive to inhibitors than the reduction of dyes, but less than that of sulfate. As an example, selenate diminished considerably the reduction rate of sulfate, though it did not affect that of sulfite in any measure. These results agreed with the observation of Postgate (*8*). Similar results were obtained for the reduction of thiosulfate.

In this case carbon dioxide also evolved, which was indicated by barium carbonate formation, being led with the bubbling nitrogen in barium hydroxide solution.

Availability of Organic Compounds as Hydrogen Donors

At first, availability for methylene blue reduction was tested. As shown in Table II. unexpectedly only a few of the compounds tested could reduce methylene blue. They were lactate, pyruvate, formate and molecular hydrogen. Especially it was noted that glucose and acetate could not reduce the dye.

TABLE II

Availability for Methylene Blue Reduction

Lactate	+	Pyruvate	+	Formate	+
Glucose	±	Glycerol	±	Ethanol	—
Acetaldehyde	±	Acetate	—	Malate	—
Succinate	—	D,L-Alanine	—	Glutamate	—
Hydrogen	+				

In the tubes: $M/15$ phosphate buffer, 2 ml.+0.01% methylene blue solution 0.5 ml.+$M/50$ substrate, 1 ml.

In the side arms: suspension, 1 ml (0.2 mg. N).

Next, the availability for sulfate reduction were tested and it was proved that also in this case only the same compounds—lactate, pyruvate, formate and hydrogen—caused the development of hydrogen sulfide from sulfate, sulfite or thiosulfate. The other compounds, which did not reduce methylene blue, also did not reduce sulfate, sulfite and thiosulfate.

The rates of hydrogen sulfide development with lactate, pyruvate and formate were shown in Fig. 1. In the cases of formate and pyruvate, the amounts of hydrogen sulfide developed were linear with time. In the case of lactate, the development of hydrogen sulfide began after a lag of 20 minutes, which can not be eliminated in spite of adding small amounts of pyruvate or formate.

The amounts of hydrogen sulfide and carbon dioxide, which were evolved with these hydrogen donors and excessive amounts of sulfate determined by Warburg manometers. The results were listed in Table III.

According to it, the ratios of hydrogen sulfide to carbon dioxide in the oxidation of lactate, and pyruvate are respectively 1 : 2.3, and 1 : 4.5.

Oxidation of lactate, pyruvate and formate, but not acetate by our strains suggests that both pyruvate and formate may be the intermediate products and acetate may be the final one of the oxidation of lactate. If it was true, the observed values of the ratios of hydrogen sulfide to carbon dioxide developed would coincide with the calculated values by Eqs. 4, 5 and 6.

and carbon dioxide, the second with lead acetate—acetic acid mixture was to indicate the amount of developed hydrogen and carbon dioxide, and the third was to do that of hydrogen. Reaction was carried out in an atmosphere of nitrogen. At the end of the incubation period, dilute sulfuric acid was tipped in reaction mixtures from the second side arms. Then carbon dioxide and hydrogen sulfide bound in the reaction mixtures were liberated. Each quanity of gases formed were calculated with the readings of three manometers.

EXPERIMENTS AND RESULTS

Reduction of Sulfate and Other Sulfur Compounds by Lactate

Some investigators reported that, besides sulfate, various inorganic sulfur compounds were reduced to hydrogen sulfide by sulfate-reducing bacteria. It is observed that in the presence of lactate or hydrogen the strain I could reduce sulfite, thiosulfate, dithionite, colloidal sulfur and tetrathionate to hydrogen sulfide, and not dithionate, trithionate and pentathionate. In the contrast with these all organic sulfur compounds tested—taurine, cysteine, cystine, cysteinic acid, benzene sulfonate, toluene sulfinate and rongalite—did not evolve any hydrogen sulfide and not absorb any hydrogen.

The quantitative relations of added sulfate, sulfite and thiosulfate to hydrogen uptakes are shown in Table I.

Table I

Hydrogen Uptake in the Reduction of Sulfate, Sulfite and Thiosulfate

Hydrogen acceptors		Hydrogen uptake	
		Found	Calculated
	μM	μM	μM
K_2SO_4	3	10.5	12.0
Na_2SO_3	2.5	6.8	7.5
,,	7.5	22.1	22.5
$Na_2S_2O_3$	2.5	9.7	10.0

These observed values were in accord with the calculated values based on the following Eqs. (1), (2) and (3).

$$H_2SO_4+4H_2=H_2S+4H_2O \quad (1)$$
$$H_2SO_3+3H_2=H_2S+3H_2O \quad (2)$$
$$H_2S_2O_3+4H_2=2H_2S+3H_2O \quad (3)$$

In the experiments with lactate as hydrogen donor the amounts of hydrogen sulfide developed were equivalent to those substrates. It is certain, therefore, that added sulfate, sulfite and thiosulfate were almost quantitatively reduced to hydrogen sulfide.

vibrios and are motile.

The pure culture of these strains were preserved in deep agar cultures. For this purpose solid medium I was used. In order to obtain large amounts of cells, we used flasks of appropriate sizes containing liquid medium II with alkaline pyrogallol plugs.

Medium I		Medium II	
Peptone	5 g.	Peptone	5 g.
$MgSO_4 \cdot 7H_2O$	1.5 g.	Sodium lactate	5 g.
Na_2SO_4	1.5 g.	$MgSO_4 \cdot 7H_2O$	1.5 g.
KH_2PO_4	0.1 g.	Na_2SO_4	1.5 g.
Agar	12 g.	KH_2PO_4	0.1 g.
Tap water	1000 ml. pH 7.0	Tap water	1000 ml. pH 7.0

Each medium was sterilized at 120° for 20 minutes. Before incubation, pH of the medium was adjusted to 7 with 1 *N* NaOH. To agar culture and some liquid cultures, it was added Mohr's salt heated in a flame. After inoculation, cultures were incubated at 37° for 48–72 hours.

Preparation of Cell Suspensions

The grown cells were harvested by centrifugation (2,500 r.p.m., 30 minutes) and washed twice with saline (0.8 per cent). Thereafter cells were suspended in *M*/15 phosphate buffer, pH 7.0. Concentrations of cell suspension were about 1 mg. N/ml.

Measurements of Activities

Measurements of Hydrogen Sulfide Formed—As described in the previous papers (*10*), reaction mixtures were incubated anaerobically, bubbling nitrogen free from oxygen, and hydrogen sulfide formed was drived away from the reaction mixtures. The amounts of the hydrogen sulfide evolved were determined by the method of St. Lorant (*11*). The experiments required preincubation of cell suspension with lactate for 1 hour, because it produced considerable amounts of hydrogen sulfide even in absence of sulfate added.

Measurements of Dehydrogenation—Usual Thunberg techniques were employed.

Measurements of Hydrogen Uptakes—Hydrogen uptake was measured by Warburg manometer in an atmosphere of hydrogen. In order to activate the hydrogenase system, cell suspension was previously incubated with hydrogen. Each flask contained cell suspension (and sometimes inhibitors) in the main chamber, sulfate or other various hydrogen acceptors in the side arm and 10 per cent KOH in the center well (*12*).

Measurements of the Amounts of Hydrogen Sulfide, Carbon Dioxide and Hydrogen Formed—They were determined by three manometers. They were provided respectively with no absorptive solution, with lead acetate—acetic acid and with potassium hydroxide in their center wells. The first manometer with no absorptive solution was used for estimating the total gas formed. Because lead acetate—acetic acid mixture (pH 3.5) absorbed hydrogen sulfide only, and potassium hydroxide did both hydrogen sulfide

The Journal of Biochemistry, Vol. 41, No. 5, 1954

BIOCHEMICAL STUDIES ON SULFATE-REDUCING BACTERIA

III. SULFATE REDUCTION BY CELL SUSPENSION

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(Received for publication, June 1, 1954)

Sulfate-reducing bacteria reduce sulfate to sulfide anaerobically at the expence of hydrogen donors and derive energy for the growth from this oxidoreduction. These organisms are generally conceived to be strict anaerobes and bear the genetic name of "Desulfovibrio." They are widely distributed in various types of soils, natural waters and seas, and occasionally give rise to extensive development of hydrogen sulfide, owing to which many agricultural and economic troubles are caused. In our country, hydrogen sulfide developed by the sulfate-reducing bacteria gives harm sometimes for rice-planting (*1*).

In 1895, Beijerinck stated in his report that the sulfate reduction in ditch water is performed by some kinds of bacteria (*2*). Van Delden isolated sulfate-reducing bacteria in pure cultures (*3*). Since then these bacteria have been studied by Elion (*4*), Baars (*5*), Starkey (6), Stephenson (*7*), Postgate (*8*), ZoBell (*9*) and others. But their investigations have been from the point of bacteriology, and the enzymatic mechanism of the sulfate reduction by these bacteria has scarcely been studied. So we intended to make this mechanism clear and studied the sulfate reduction by use of cell suspension.

In this paper it is concerned mainly with the descriptions about general features of the sulfate reduction by sulfate-reducing bacteria.

MATERIALS AND METHODS

Strains Used and Cultivation

Usually *Desulfovibrio desulfuricans*, strain I, was employed. This strain was isolated in pure culture from soil of paddy field in Miyazaki prefecture by H. Iwasaki and was studied by T. Hirose in our laboratory. Another strain II, isolated from ditch mud in Chiba, was used in some experiments. Both strains are strictly anaerobic

were on the whole less active than the cationic surface active agents. As in the case of the latter substances, the inhibitory action of these products cannot be accounted for solely on the basis of surface tension depression.

The author wishes to gratefully acknowledge the guidance and advice of Dr. C. Richard Smith of Barlow Sanatorium, Dr. Milton C. Kloetzel of the Department of Chemistry, University of Southern California, and Dr. Cyrus O. Guss of the Department of Chemistry, Colorado Agriculture and Mechanical College.

Thanks are also due to the various companies which submitted their products for this study, and to Mrs. Annie Fry and Mrs. Annie Pearl Lockett for their indispensable aid.

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10^{-4} per cent. He also reported that two other alkyl sulfates with 10 to 16 carbon alkyl chains prevented growth at 10^{-3} per cent.

In our studies with the alkyl taurines (Table III) the palmitylmethyl derivative was found to be more effective than the lauryl derivative. It was also more effective than the longer chained, but unsaturated, oleylmethyl taurine.

The bacteriostatic activity of the nonionic ethylene oxide reaction products does not appear to be particularly influenced by the number of the ethylene oxide units present in the molecule. Rosin Amine D ethylene oxide reaction product with four ethylene oxide units was just as active as the product with twelve ethylene oxide units.

Nonionic Tween 80, an important constituent of Dubos medium for culturing of tubercle bacillus, under our conditions of study was found to be completely bacteriostatic at 1 : 10^4 dilution. Dubos and Middlebrook (*5*) also reported that commercial Tween 80, as we have used it, is inhibitory to the growth of the tubercle bacillus. However, their more detailed investigations indicated that the toxicity of Tween 80 was due to the small concentration (reported to be 0.6 per cent) of free oleic acid present in the product, and that the esters *per se* were non-toxic.

With one exception (No. 12) all solutions with surface tensions of 40 dynes per centimeter or below gave complete growth inhibition. Some solutions with surface tensions of 50 or more were also found to be completely inhibitory.

SUMMARY

Twenty anionic and thirteen nonionic surface active agents were tested for bacteriostatic activity against *Mycobacterium tuberculosis* H37Rv in Proskauer-Beck liquid synthetic medium (Youmans' modification). Most were completely bacteriostatic at 1 : 10^4 dilution; a few, at 1 : 10^5; while none was completely inhibitory at 1 : 10^6 dilution. These products

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15. Division of Medicinal Chemistry, The Squibb Institute of Medical Research
16. Synthetic Chemicals Incorporated
17. Victor Chemical Works
18. Jacques Wolfe & Company
19. Wyandotte Chemicals Corporation

*Trade name was omitted at the request of the supplier.

4) The dilutions listed are the dilutions of the surface active agents.

TABLE VII

Bacteriostatic Effect of Nonionic Surface Active Agents on Mycobacterium tuberculosis H37Rv

Etylene Oxide Reaction Products

Surface active products3)	Dilution4)	pH	S.T.	I.I.
28. Hydroabietyl alcohol ethylene oxide reaction product (X5520-15-4, 10)	$1:10^4$	7.4	37	10
	$1:10^5$	7.4	48	8
	$1:10^6$	7.1	56	1
29. Diterpene diphenol ethylene oxide reaction product with 2 ethylene oxides (X5503-48-B, 10)	$1:10^4$	7.5	43	10
	$1:10^5$	7.2	47	1
	$1:10^6$	7.0	55	1
30. Rosin amine D ethylene oxide reaction product with 4 ethylene oxides (X-5603-32, 10)	$1:10^4$	7.1	36	10
	$1:10^5$	6.8	50	10
	$1:10^6$	6.9	62	0
31. Rosin amine D ethylene oxide reaction product with 12 ethylene oxides (X5603-29, 10)	$1:10^4$	7.1	39	10
	$1:10^5$	7.0	36	10
	$1:10^6$	7.1	52	1
32. Rosin substituted phenol ethylene oxide reaction product with 20 ethylene oxides (X5503-50-B, 10)	$1:10^4$	7.0	43	10
	$1:10^5$	7.2	49	3
	$1:10^6$	7.1	58	0
33. Menthylphenol ethylene oxide reaction product (X5503-32-A, 10)	$1:10^4$	7.0	36	10
	$1:10^5$	7.1	40	10
	$1:10^6$	7.0	47	1

(Footnotes to the tables)

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3) In parentheses after each compound name and composition are given the trade name or the supplier's designation of the product and a number referring to the source of the product as follows:

1. Alox Corporation
2. Alrose Chemical Company
3. American Cyanamid Company
4. Atlantic Refinery Company
5. Atlas Powder Company
6. Ciba Company, Inc.
7. Dewey and Almy Chemical Company
8. E. I. du Pont de Nemours & Company
9. General Dyestuff Corporation
10. Hercules Powder Company
11. Kem Products Company
12. Monsanto Chemical Company
13. Proctor & Gamble
14. Rohm & Haas Company

Table V

Bacteriostatic Effect of Nonionic Surface Active Agents on Mycobacterium tuberculosis H37Rv

Polyether Alcohols

Surface active product[3]	Dilution[4]	pH	S.T.	I.I.
21. Alkylarylpolyether alcohol 33% sol. (Triton X-30, 14)	$1:10^{4}$	7.5	33	10
	$1:10^{4.3}$	7.5	36	10
	$1:10^{4.6}$	7.2	39	10
	$1:10^{4.9}$	7.4	47	4
	$1:10^{5.2}$	7.6	51	2
22. Alkylarylpolyether alcohol (Triton X-100, 14)	$1:10^{4}$	6.9	32	10
	$1:10^{5}$	6.9	44	3
	$1:10^{6}$	6.9	59	2
23. Nonylphenoxydecapolyethylene glycol (N *Se*M P'c NPE-10, 4)	$1:10^{4}$	7.0	36	10
	$1:10^{5}$	7.1	41	2
	$1:10^{6}$	7.0	55	1

Table VI

Bacteriostatic Effect of Nonionic Surface Active Agents on Mycobacterium tuberculosis H37Rv

Fatty Acid Esters

Surface active product[3]	Dilution[4]	pH	S.T.	I.I.
24. Sorbitan monooleate polyoxyalkylene derivative 97–100% (Tween 80, 5)	$1:10^{4}$	6.9	42	10
	$1:10^{5}$	7.1	44	1
	$1:10^{6}$	7.0	54	0
25. Fatty acid esters (Soluble Castor oil, 11)	$1:10^{4}$	6.9	35	10
	$1:10^{5}$	6.8	47	10
	$1:10^{6}$	6.9	58	0
26. Nonaethylene glycol distearate (Nonisol 310, 2)	$1:10^{4}$	6.7	39	10
	$1:10^{5}$	6.7	52	10
	$1:10^{6}$	6.6	52	0
27. Nonaethylene glycol monostearate (Nonisol 300, 2)	$1:10^{4}$	6.9	40	10
	$1:10^{5}$	7.2	47	5
	$1:10^{6}$	7.2	55	2

alkylarylsulfonates have been reported to be only partially inhibitory to the growth of the non-virulent Novy strain of tubercle bacillus at a dilution of 1 : 10,000 (*3*). Of the three Tergitol penetrants, sodium sulfate derivatives of 2-ethylhexanol, 7-ethyl-2-methylundecanol-4-, and 3,9-diethyltridecanol-6, tested by Drea (*4*) against *M. tuberculosis* H37, the last was reported to be the most effective, and prevented growth at

Table III

Bacteriostatic Effect of Anionic Surface Active Agents on Mycobacterium tuberculosis H37Rv

Salts of Fatty Acid Amide Sulfonates or Sulfates

	Surface active product[3]	Dilution[4]	pH	S.T.	I.I.
12.	Sodium palmitylmethyltaurine & sodium salts (*, 9)	$1:10^4$	6.9	31	10
		$1:10^5$	7.0	39	8
		$1:10^6$	7.0	59	0
13.	Sodium oleylmethyltaurine & sodium salts (*, 9)	$1:10^4$	6.8	31	10
		$1:10^5$	6.8	48	0
		$1:10^6$	6.7	64	10
14.	Sodium-N-lauroyl-2-amino-ethylsulfate 31%, 69% inorganic salts & water (Alrosene 31, 2)	$1:10^4$	7.3	34	10
		$1:10^5$	7.3	50	2
		$1:10^6$	7.1	55	0
15.	N-(α-alkylcinnamoyl)-2-amino-ethylsulfonic acid, alkyl=8 to 14 C (MC-609, 15)	$1:10^4$	7.0	34	10
		$1:10^5$	6.9	43	7
		$1:10^6$	7.0	51	1

Table IV

Bacteriostatic Effect of Miscellaneous Anionic Surface Active Agents on Mycobacterium tuberculosis H37Rv

	Surface active product[3]	Dilution[4]	pH	S.T.	I.I.
16.	Sodium sulfoethyloleate & inorganic salts (*, 9)	$1:10^4$	6.9	32	10
		$1:10^5$	7.0	44	0
		$1:10^6$	6.9	48	0
17.	Sodium alkylsulfonate (MP-189 Lot 5C, 8)	$1:10^4$	6.9	41	10
		$1:10^5$	6.9	48	0
		$1:10^6$	7.0	65	0
18.	Fatty alcohol sulfate (Dreft, 13)	$1:10^4$	6.9	40	10
		$1:10^5$	6.9	53	0
		$1:10^6$	6.9	65	0
19.	Dioctyl sodium sulfosuccinate 100% (Aerosol OT, 3)	$1:10^4$	6.9	31	10
		$1:10^5$	6.8	44	0
		$1:10^6$	6.8	56	0
20.	Oxidation product of low mole. wt. hydrocarbons (Alox 828, 1)	$1:10^4$	6.9	56	0
		$1:10^5$	6.7	56	0
		$1:10^6$	6.8	57	0

While sodium xylene sulfonate was completely ineffective even at $1:10^4$ dilution, sodium decylbenzene sulfonate completely inhibited the growth of the tubercle bacillus at $1:10^5$ dilution. Previously various

TABLE IV

Inhibition of Reduction of Sulfate, Thiosulfate and Dyes

Inhibitory reagents	Concentration (M)	Hydrogen uptake				H_2S development			Dehydrogeneration of lactate
		SO_4^{--}	SO_3^{--}	$S_2O_3^{--}$	Phenosafranine	SO_4^{--}	SO_3^{--}	$S_2O_3^{--}$	
KCN	10^{-4}	69	0						
	1.25×10^{-4}	69	14		2				
	2.5×10^{-4}					97	10		
	1.25×10^{-3}	100	52	73	27				
	2.5×10^{-3}						99		0
	1.25×10^{-2}		100						
Na_2SeO_4	2.5×10^{-4}	80	0	15	6	97	0		12
NaN_3	2.5×10^{-4}	74							
	1.25×10^{-3}		52	7		56			
	1.25×10^{-2}	100	97		25	90	67		75
$NaAsO_2$	2.5×10^{-4}	100	86	36	0	100	100	100	0
$NH_2OH\cdot HCl$	2.5×10^{-4}	100	61	43	3	100			
Na_2WO_4	10^{-3}	72	21	70	32				
	10^{-2}	100							

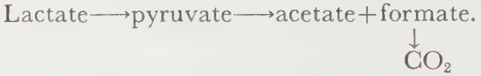
Experimental conditions:

Hydrogen uptake: In main chambers of vessels of Warburg manometers were contained cell suspension 2 ml., $CdCO_3$ suspension 1 ml., solution of inhibitors 0.5 ml., in side arms hydrogen acceptor solution 0.5 ml. (K_2SO_4, Na_2SO_3, and $Na_2S_2O_3$; 0.05 M, phenosafranine; 0.01 M), and in center well KOH 10% 0.2 ml. Gas phase; hydrogen. Temperature; 37°. Degree of inhibition was given in percentage of decrease of hydrogen uptake in first 20 minutes.

H_2S development: Cell suspension (containing 0.017 M lactate and 0.02 M phosphate buffer) 3 ml. and Na_2SO_4, Na_2SO_3 or $Na_2S_2O_3$ 0.01 M 1 ml. and inhibitor 1 ml. Temperature: 37°.

Dehydrogenation of lactate: Thunberg tubes were employed, containing lactate 0.1 M 0.5 ml., methylene blue 0.01% and inhibitor 1 ml. in the tubes and cell suspension in side arms. Temperature: 37°. Degree of inhibition was given in percentage of the decrease of reciprocal of decolorization time in minutes.

From the observations that our strains oxidized lactate, pyruvate formate and not acetate, acetate seems to be the final product in the oxidation of these compounds. The observed ratios of hydrogen sulfide to carbon dioxide formed in the oxidation of these compounds were approximate to the calculated values. So the oxidations of these compounds were supposed to proceed successively according to the following schema:



$$\text{Lactate} \longrightarrow \text{pyruvate} \longrightarrow \text{acetate} + \underset{\substack{\downarrow \\ CO_2}}{\text{formate}}.$$

In the absence of hydrogen acceptors our strain I decomposed formate and pyruvate, and developed molecular hydrogen and carbon dioxide. The ratio of the amounts of the gases indicated that the following reactions might take place:

$$HCOOH = H_2 + CO_2,$$
$$CH_3COCOOH + H_2O = CH_3COOH + H_2 + CO_2.$$

Postgate, however, reported that his strain dismuted pyruvate to acetate and alcohol (*13*), and our result differed from his. It may be that our strain is different from his.

From the fact that reductions of sulfate, sulfite and thiosulfate are more sensitive to various inhibitors than reduction of dyes, it may be conceived that some specific enzyme system, which suffered inhibition by these agents, play a role in the reduction of sulfate, sulfite and thiosulfate, coupling with hydrogenase and dehydrogenases.

Compared with the sulfite reduction, the sulfate reduction is more inhibited by various inhibitors, especially by selenate, as indicated early by Postgate. This fact shows that the sulfate reduction requires more complicated enzyme system than the sulfite reduction. Although experiments for sulfite detection in the course of sulfate reduction in various conditions were tried with no success, it is probable that sulfite may be an intermediate in the sulfate reduction and an enzyme reducing sulfate to sufite, may be sensitive to selenate. In the case of thiosulfate reduction, similarly sulfite accumulation could not be observed, perhaps because of a larger rate of reduction of sulfite than that of thiosulfate, but, as the extract of the strain was found to have an ability to reduce thiosulfate to sulfite with molecular hydrogen, sulfite should be an intermediate of thiosulfate reduction. Possibility that polythionates occur as intermediates in the process of the reduction can be eliminated.

These conclusions, however, are not conclusive, as the experiments were tried only with the living cell suspensions, in the case of which many unknown complex factors, *e.g.* permeability of the cell membrane, should take part in the reaction. For the establishment of the mechanism of the sulfate reduction experiments in the cell-free state is required.

SUMMARY

1. Sulfate reduction by cell suspension of sulfate-reducing bacteria were studied by measuring developments of hydrogen sulfide and absorption of molecular hydrogen. Our strain reduced sulfate, sulfite, thiosulfate, dithionite, colloidal sulfur and tetrathionate to hydrogen sulfide at the expense of appropriate hydrogen donors. Dithionate, trithionate, pentathionate, taurine, cysteine, cystine, cysteic acid, benzene sulfonate, toluene sulfinate, and rongalite were not reduced. It was observed that added sulfate, sulfite, and thiosulfate are almost quantitatively reduced to hydrogen sulfide.

2. As hydrogen donors, lactate, pyruvate, formate and molecular hydrogen were utilized for sulfate reduction, as well as dehydrogenation of various dyes. Glucose, glycerol, malate, succinate, citrate, alanine and acetate did not appear to be utilized. In absence of hydrogen acceptors such as sulfate, the strain decomposed pyruvate and formate, and developed molecular hydrogen.

3. The reduction of sulfate, sulfite and thiosulfate were more inhibited by various inhibitors such as cyanide, arsenite, azide than the reduction of various dyes.

The authors wish to thank Dr. T. Soda for his kind guidance and Dr. H. Tamiya for valuable advice throughout this investigation. The expenses for the work were aided partly by the Scientific Research Funds from the Ministry of Education.

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STUDIES ON THE ANTERIOR PITUITARY GONADOTROPHIN

II. PURIFICATION AND CRYSTALLIZATION OF BEEF INTERSTITIAL CELL STIMULATING HORMONE (ICSH)

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Gonadotropic hormones may be divided into two main groups, one of which is derived from pituitary and the other from living chorionic tissue. Biological studies of pituitary gonadotrophin reveal certain interesting characteristics which may be taken to classify them into two groups: one is concerned mainly with the stimulation of follicle growth in the ovaries and the increase of spermatogenic activity in the male, and the other with the transformation of follicles to corpora lutea in the ovaries, the stimulation of interstitial cells, and the enlargement of the secondary sex glands in the male. The former is called follicle-stimulating hormone (FSH) and the latter interstitial cell-stimulating hormone (ICSH). It is now established beyond any doubt that two hormones (FSH and ICSH) are present in the pituitary extract and are responsible for the gonadotropic activity.

Interstitial cell-stimulating hormone (ICSH) of the anterior pituitary was obtained by Li *et al.* (*1*) and Chow *et al.* (*2*) in 1940 from sheep and pig gland, respectively. Both preparations behaved as a single protein in electrophoresis, ultracentrifuge and solubility studies. It will be seen that the same hormones isolated from sheep or pig pituitary are not identical substances although their biological characteristics are very similar.

Previously, we reported the isolation and purification of this hormone from beef gland and obtained ICSH as a crystalline substance (*3*). In this present report, beef anterior pituitary is extracted with 40% methanolic acetate buffer solution (pH 5.0) and crude ICSH is precipitated with methanol. Then, ICSH is purified with rivanol treatment and acetone fractional precipitation.

EXPERIMENTAL

Extraction and Purification—100 g. of acetone-desiccated beef anterior pituitary tissue were extracted with 1 *l.* of 40 per cent methanolic acetate buffer solution at pH 5.0 and ionic strength of 0.15 over a period of 24 hours with constant stirring in a cold room. After centrifugation, the residue was re-extracted similarly. The supernatants were combined and filtered through coarse folded filter paper. The clear filtrate was brought to 85 per cent methanol concentration by the addition of cold methanol. The precipitate formed was extracted with distilled water. To this extract (pH 6.8) 1 per cent solution of rivanol (2-ethoxy-6,9-diamino acridinium lactate) was added slowly until no more precipitate was formed. The rivanol precipitate was centrifuged off and the yellow supernatant was brought to 90 per cent acetone concentration. The precipitate thus formed was dissolved in water again and brought to 33 per cent acetone concentration and stored in refrigerator overnight. This precipitate was biologically inactive, so centrifuged off and the supernatant was brought to 50 per cent acetone concentration gradually and stored in refrigerator for a few days. Then ICSH was crystallized almost completely. These crude crystals obtained as above were dissolved in a small volume of water and recrystallized several times. All process described above were carried out below 5°. The pure crystals thus obtained are prism as shown in Fig. 1. The procedure mentioned above is summarized in the following chart:

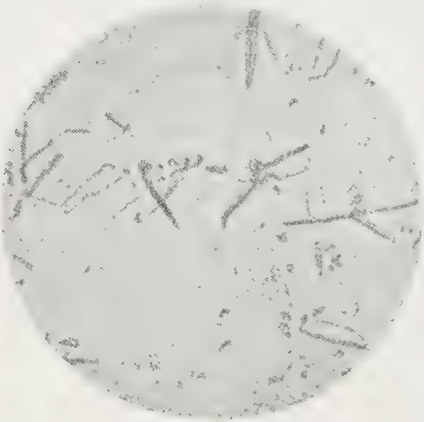

Fig. 1. Crystalline ICSH (×100)

From 50 g. of beef anterior pituitary glands there were obtained 20 mg. of pure ICSH crystals. The yield of each fraction was summarized in Table I.

Biological Assay—The physiological activity of each fraction was examined by the ovarian hyperemia test with immature female rat and data were shown in Table I.

0.025 mg. of this beef ICSH crystal caused apparently positive result in ovarin hyperemia test with immature female rat.

Chemical Data—The beef ICSH crystal was examined with Tiselius electrophoresis apparatus. In phosphate buffer at pH 8.0, ionic strength 0.1, this crystal showed homogeneous single peak after 180 minutes migration.

The solution of ICSH crystal shows strong biuret, Millon and Molish reactions and seemed to be apparently glucoprotein. The analytical values are as follows:

N: 13.15%   Na: 0.77%

Rivanol Method for the Purification of Beef ICSH

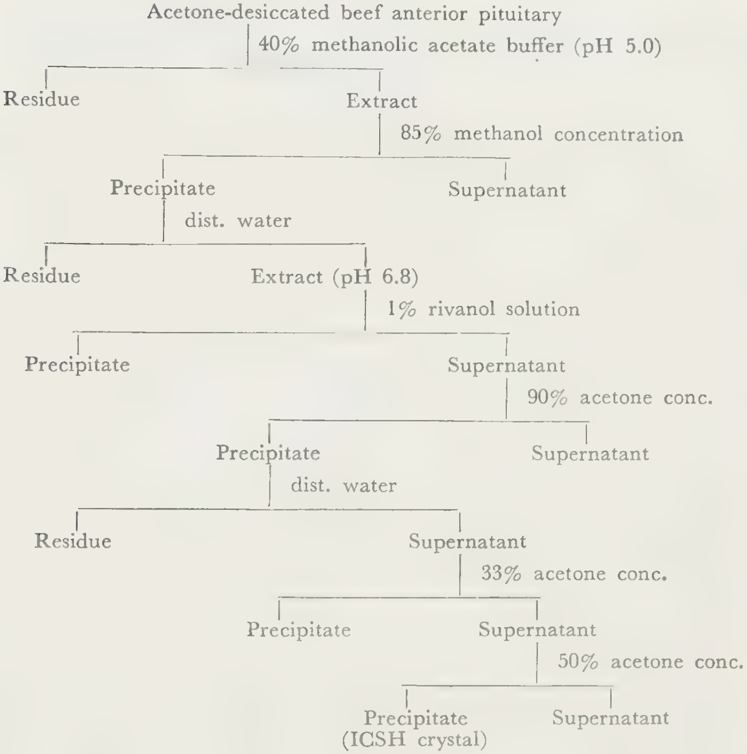

TABLE I

Yield and Physiological Activity of Each Fraction in Methanolic Acetate Fractionation

Fraction	Yield*	Activity (M.E.D.)**
	mg.	γ
Methanol precipitate	350	150
Acetone precipitate after rivanol treatment	250	50–80
33% acetone precipitate	50	—
50% acetone precipitate	20	25–50
75% acetone precipitate	25	?

* From 50 g. of acetone desiccated beef anterior pituitary gland.

** Ovarian hyperemia test with immature female rat.

DISCUSSION

The interstitial cell-stimulating hormone of sheep and pig was obtained by Li *et al.* and Chow *et al.* previously, but about the beef ICSH, there was no report of purification up to this time.

Now we obtained this beef hormone in pure and crystalline form with rivanol purification method. By this methanolic acetate method, the yield of substance is half as compared with ethanolic acetate method reported previously (*3*) but the activity is twice. The Na content is also less and the crystal is very minute than that of ethanolic acetate method. When this crystal was dialyzed, it gave only amorphous powder, so it seemed that some metals are indispensable for the crystallization of ICSH.

The method of eliminating the inactive acidic protein by precipitation with basic substance and purifying the protein hormone is applied by Claesson *et al.* (*4*) in the purification of pregnancy urine gonadotropin. They used protamine as a basic substance, and with ethanol fractional precipitation obtained this hormone in crystalline form.

But the use of the basic dye as a purification agent is very rare. We studied several dyes other than rivanol and recognized that rivanol, acriflavin and fuchsin are also available for the purification of this hormone, but rivanol is the most effective.

SUMMARY

40 per cent methanolic acetate (pH 5.0) was used for the extraction of ICSH from beef anterior pituitary. Homogeneous ICSH crystal was obtained by the rivanol treatment and acetone fractionation procedure.

The authors wish to express their gratitude to Prof. S. Akabori and Dr. K. Takeda for their kind guidance and encouragements through this investigation and to Mr. K. Ikeda for his much technical assistance.

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ON FORMIC DEHYDROGENASE OF BEAN SEED EMBRYO IN THE GERMINATION STAGE*

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It has been reported that concerning dehydrogenase pattern there exists a distinct difference between the reserve tissues (cotyledon) and the growing ones of germinating seed of *Vigna sesquipedalis* (*1*). The former is completely deprived of the citric dehydrogenase activity and contains a high activity of formic dehydrogenase, whereas the latter excels in the citric enzyme activity and is rather poor in formic dehydrogenase. A function of citric dehydrogenase in these embryo tissues has already been discussed in detail (*1*, *2*). The present study was performed with a view to get informations about a) the physiological meaning of formic dehydrogenase in seed embryo of *Vigna sesquipedalis*, particularly in the pulpy tissues of cotyledon, and b) the chemical nature of plant formic dehydrogenase, in which many authors have long been interested because of its high sensitivity to metal combining substances, *e.g.* cyanide and azide (*4*, *7*). This work has been undertaken for the most part along the line of the Davison's studies on pea and bean formic dehydrogenases (*5–7*), and as will be seen below the results obtained are not always coincident with, but sometimes even completely contradictory to hers.

MATERIALS AND METHODS

Bean Seeds—Seeds of *Vigna sesquipedalis* harvested in summer 1953 in the farm of our laboratory were used. Details of the methods of seed germination and tissue extract preparation have been described in our previous paper (*1*). As the O-day-old material, the seeds soaked in tap water for 5 hours at 30° were employed.

Dehydrogenase Activity—A detailed account of the procedure employed has also been presented in the previous report (*1*). Time spent for 70 per cent reduction of added methylene blue (Mb) was measured with evacuated Thunberg tubes immersed in a thermostat at 30°.

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* As for the definition of the germination stage, see Oota *et al.* (*1–3*).

O_2 Uptake—Warburg's manometric method was used for the determinations (*cf. 8*). All the experiments were carried out at 30°.

CO_2 Output and Uptake—These were also measured "directly" (*8*) using Warburg's respirometers at 30°. $NaHCO_3$ at a final concentration of 0.017 *M* was added to the experimental medium for the sake of pH regulation (pH 7.4), and the gas space was filled with 5 vol. per cent CO_2 in N_2. Before use, CO_2 from an ordinary iron cylinder was washed with saturated $CuSO_4$ and $KHCO_3$ solutions. Nitrogen gas, taken also from commercial cylinder, was purified with alkaline pyrogallol and $CuCl$-NH_4Cl solutions succesively, and then by flowing through a combustion tube containing reduced copper powder (at 500°).

Protein Nitrogen—The difference between total N and inorganic N estimated for a given sample was considered as the amount of protein N of the sample in question. For the N estimation, the method of Levy and Palmer modified by Yagi (*9*) was used.

Dry Weight—Samples were dried at $100\pm5°$ until constant weight was attained. The dry matters were cooled in a dessicator over $CaCl_2$ before weighing.

Old Yellow Enzyme—This was prepared from dried brewer's yeast by the method of Warburg and Christian (*10*). The purification procedure was conducted until the chloroform step.

Diphosphopyridine Nucleotide (DPN)—The method of Williamson and Green (*11*) was employed for the preparation of crude DPN from brewer's yeast. Reduced diphosphopyridine nucleotide (DPN.2H) was prepared by the method of Lehninger (*cf. 12*).

Adenosine Triphosphate (ATP)—The method of Szent-Györgyi (*13*) was employed using fresh skeletal muscle of a dog killed by $MgSO_4$-injection (*14*).

Reagents—Except α-ketoglutaric acid which was prepared by the method described in Umbreit *et al.* (*8*), reagents used were purchased in the market. CO was prepared from formic acid and H_2SO_4, and washed with alkaline pyrogallol solution before use.

RESULTS

Change in the Formic Dehydrogenase Activity of Germinating Seed—The formic dehydrogenase activity in the water extracts of respective organs excised from the embryos of various ages is shown in Fig. 1., the data for organs other than epicotyl being derived from Oota *et al.* (*1*). The highest activity is found in cotyledon, and all tissues of the seedling part, inclusive of plumules, epicotyl, hypocotyl, and radicle, are found to possess only low activity. It is noteworthy that the gaseous conditions of tissues seem to have influences on the there existing enzyme activity. It would be needless to say that considerably anaerobic conditions may prevail in the pulpy tissues of cotyledon, while the growing tissues would be subjected to exceedingly aerobic conditions.

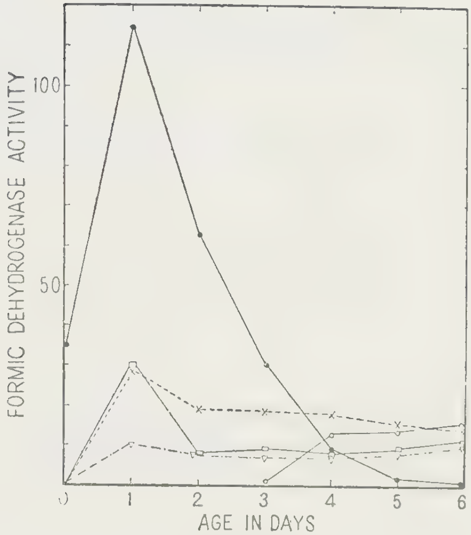

FIG. 1. Change in the formic dehydrogenase activity of various organs of *Vigna sesquipedalis* in the germination stage.

Standard system; Tissue extract (*cf.* Oota *et al.* (*1*)), 1 ml.: formate (*M*/10), 1 ml.: phosphate buffer (*M*/5, pH 7.4), 2 ml.: Mb (1 : 100,000), 1 ml. Final volume; 5 ml. Thunberg tubes: 30°.

The activity is expressed in terms of reduction rate, 100 (1/T−1/T′)/W, where T, and T′ denote decoloration time (min.) of experimental and control runs, respectively, and W dry weight of the tissue extract employed (in g.).

—●—●—	Cotyledon
—□—□—	Radicle
- -×- -×- -	Hypocotyl
—○—○—	Epicotyl
—·—▽—·—▽	Plumule

The effect of aeration on formic dehydrogenase was then examined. Two hundred 1-day-old germs were homogenized with 200ml. of 0.1*M* Na$_2$HPO$_4$ in a Waring blendor. The homogenate was squeezed through muslin and centrifuged for 20 minutes at 700× *g*. The supernatant (about 200 ml.) was mixed with 120 g. of (NH$_4$)$_2$SO$_4$ (about 0.8 saturation). The precipitate collected on kieselguhr by suction was dissolved in distilled water. Remaining kieselguhr was removed by a brief centrifugation. The aliquots of the supernatant were bubbled with 2 *l.* each of CO$_2$, N$_2$, O$_2$, and air for 30 minutes, respectively, and then assayed for the formic dehydrogenase activity (Table

I). A poisoning effect of both O_2 and air is clearly seen, while neither CO_2 nor N_2 exerts any remarkable inhibition. Bacterial formic dehydrogenase is reported to be likewise inactivated by shaking with air in the presence of formate, the inactivation being enhanced by increasing the O_2 pressure (*15*).

It was revealed that the inactivation caused by the O_2 bubbling could be removed by the addition of such reducing agents as cysteine and ascorbic acid (Table II). The recovery of activity was complete by cysteine but only partial by ascorbic acid.

TABLE I

Effects of Gases on the Formic Dehydrogenase Activity

Bubbled with:	−		CO_2	N_2	O_2	Air
Formate added:	−	+	+	+	+	+
Mb-reduction time (*min.*)	>100	16.2	18.2	18.6	29.5	25.1

Standard system ; Enzyme preparation (see the text), 1 ml. : formate ($M/10$), 1 ml. : phosphate buffer ($M/10$, pH 7.4), 1 ml. Mb (1 ; 10,000), 1 ml. : DPN (1 mg./ml.), 1 ml. Final volume ; 5 ml. Thunberg tubes : 30°.

TABLE II

Effects of Reducing Agents on the Formic Dehydrogenase Activity

O_2 treatment	−			−			+			+		
Formate	−			+			−			+		
Cysteine ($M/300$)	−	+	−	−	+	−	−	+	−	−	+	−
Ascorbic acid ($M/180$)	−	−	+	−	−	+	−	−	+	−	−	+
Mb-reduction time (*min.*)	>150	>150	>150	16.2	14.1	16.0	>150	>150	>150	29.5	15.3	25.0

Standard system ; Enzyme preparation (the same as used in Tab. I), 1 ml. : formate ($M/10$), 1 ml. : phosphate buffer ($M/10$, pH 7.4), 1 ml. : Mb (1 ; 10,000), 1 ml. : DPN (2 mg./ml.), 0.5 ml. : cysteine ($M/30$) or ascorbic acid ($M/18$), 0.5 ml. Final volume ; 5 ml. Thunberg tubes : 30°.

Partial Purification of Formic Dehydrogenase—Several attempts of purification of plant formic dehydrogenase have been reported. Adler and Sreenivasaya (*4*), and Mathews and Vennesland (*16*) extracted formic dehydrogenase from swelled peas with Na_2HPO_4 and concentrated it by $(NH_4)_2SO_4$ treatment. Davison (*7*) partly purified the enzyme of bean meal by successive fractionations with $(NH_4)_2SO_4$ and adsorption on calcium phosphate gel.

Taking into consideration the aerophobic nature of the enzyme just revealed, the following extraction method was introduced in further experiments on the bean formic dehydrogenase.

Two hundred g. (fresh weight) of 1-day-old embryos were homogenized with 200 ml. of 0.1 M Na_2HPO_4 by a Waring blendor placed in CO_2 atmosphere, and allowed to stand for 1 hour. The homogenate was squeezed through muslin, centrifuged for 20 minutes at $1,100 \times g$, the acidity of the supernatant was adjusted with NaOH to pH 7.4 (*Preparation I*) and filtered through kieselguhr by suction. To the resultant clear filtrate (*Preparation II*) was added 120 g. of $(NH_4)_2SO_4$. The precipitate was filtered through kieselguhr in CO_2 atmosphere and the residue was rubbed up with 20 ml. of 0.03 M Na_2HPO_4 in a mortar and centrifuged for 3 minutes at $1,100 \times g$. To the supernatant (*Preparation III*) was added one drop of toluene and mixed well so as to "screen out" formic enzyme out of the coexisting other enzyme (*cf. 17*). Toluene was not replaceable by butanol or ether (*cf. 18*). The mixture was allowed to stand at 30° for 2 hours and centrifuged for 10 minutes at $1,100 \times g$. The supernatant was placed in a cellophane sack and dialysed against 0.01 M Na_2HPO_4 for 5 hours, and centrifuged for 10 minutes at $1,100 \times g$. The supernatant thus obtained (*Preparation IV*) could be kept *in vacuo* in an ice box without any remarkable loss of activity for a few days. The activity and the yield at each stage of the purification procedure are given in Table III.

Instead of avove mentioned anaerobic conditions, the addition of cysteine at a

TABLE III

Activity and Yield of Formic Dehydrogenase at Various Steps of Purification

Preparation	Volume (*ml.*)	Protein-N (*mg./ml.*)	Reduction time (*min.*)		Enzyme unit	Total enzyme unit (relative value)
			Formate add (T)	No formate (T′)		
I	200	4.18	18.5	32.0	0.00119	1.00
II	200	0.59	75.0	159.0	0.00262	0.31
III	15	2.25	14.0	>300.0	0.00695	0.24
IV	16	1.65	16.5	>300.0	0.00803	0.21

Standard system; Enzyme preparation (the same as used in Tab. I), 1 ml.: formate (M/10), 1 ml.: phosphate buffer (M/10, pH 7.4), 1 ml.: Mb (1; 10,000), 1 ml.: DPN (2 mg./ml.), 0.5 ml.: old yellow enzyme (4 mg./ml.), 0.5 ml. Final volume; 5 ml. Thunberg tubes: 30°.

Enzyme unit is calculated by the following equation: $0.313 \times 0.7\ (1/T - 1/T')$/mg. protein-N [Time (in minute) for 70 per cent reduction of 0.313 μM Mb added was measured. Reduction time greater than 300 minutes was considered as infinite.]

final concentration of $M/500$ prior to the dialysis step was also found to be satisfactory for the enzyme purification.

Besides the formic enzyme activity, weak catalase and very weak peroxidase activities were detected in *Preparation IV*. Under an ocular-spectroscope the preparation showed the reduced cytochrome bands at 590 and 557 mμ in the absence as well as in the presence of added hydrosulphite. On addition of pyridine the band of pyridine haemochromogen at 557 mμ was produced.

Oxidation Pattern for Formate—Plant formic dehydrogenase is said to be DPN-specific

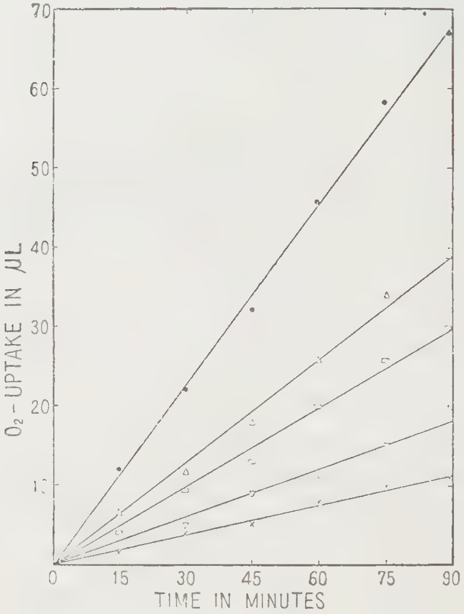

Fig. 2. Oxidation pattern for formate.

Standard system; *Preparation IV*, 1.4 ml.: formate (0.5 *M*), 0.3 ml.: phosphate buffer (0.5 *M*, pH 5.9), 0.4 ml.: Mb (M/1,000), 0.3 ml.: DPN (5 mg./ml.), 0.3 ml.: old yellow enzyme (10 mg./ml.), 0.3 ml.: Final volume; 3.0 ml. Warburg respirometers: 30°.

—●—●— Complete system

—△—△— Without Mb

—□—□— Without yellow enzyme

—▽—▽— Without DPN

—×—×— Without formate

(*4*, *7*, *19*), and to be able to couple with the cytochrome system not directly but *via* flavin enzyme (*4*, *7*). It was found to be also the case for the present material. As shown in Fig. 2, the complete system consisting of *Preparation IV*, formate, DPN, old yellow enzyme, and Mb can consume oxygen vigorously, while the absence of any one of the components lowers the rate of oxygen uptake markedly. Above all the omission of DPN causes most drastic effect.

Carrier-linked Dismutation—Working with pea or bean preparations, Davison (*7*) has found that the oxidation of formate can be coupled with the reduction of acetaldehyde or α-ketoglutarate with the intermediation of DPN. This is also the case for the present crude preparation (Tables IV and V). Any omission from the complete system consisting of cotyledon juice, formate, acetaldehyde (or α-ketoglutarate+ammonium salt), and DPN causes remarkable diminution in the CO_2 output. Davison (*6*) has also confirmed with her pea preparation that the formate oxidation can couple with the fumarate reduction. No CO_2 output, however, was observed with the crude preparation similar to that used in Table IV in the presence of fumarate as well as formate: further addition of DPN together with any one of possible intermediate H-carriers, *i.e.* old yellow enzyme, araboflavin, Mb, crystal violet, or nile blue, was proved to be incapable of evoking the coupling in question. This point should be investigated in future: one possibility may be that fumarate reductase is absent in the present material.

TABLE IV

Coupling between Formic and Alcohol Dehydrogenases

Omission from reaction mixture	No omission	Formate	DPN	Acetaldehyde
CO_2 output $\mu l./hr./g.$ juice (dry weight).	417	37	157	24

Standard system; Cotyledon juice,* 1.5 ml.: formate (0.4 *M*,) 0.8 ml.: phosphate buffer (*M*/10, pH 7.4), 0.5 ml.: DPN (5 mg./ml.) 0.9 ml.: acetaldehyde (0.4 *M*), 0.8 ml.: $NaHCO_3$ (0.17 *M*), 0.5 ml. Final volume; 5 ml. Gas room; 5 vol. % CO_2 in N_2. Warburg respirometers: 30°.

* Cotyledons (fresh weight 20 g.) removed from 1-day-old embryos were homogenized with 30 ml. of 0.2 *M* phosphate buffer (pH 7.4). The homogenate was squeezed through muslin, centrifuged for 2 min. at 130×*g*. The supernatant was adjusted to pH 7.4 with NaOH before use.

In addition to these DPN-linked dismutations, formic dehydrogenase of cotyledon was found to be capable of coupling with nitrate reductase that is also found to exist in the tissue concerned (*cf.* *20*) (Table VI). In this case the addition of DPN, old yellow enzyme, and Mb was needed.

CO_2 Fixation—Attempts to reverse the decarboxylating reaction of formic dehydrogenase were unsuccessful. A system consisting of *Preparation IV* (1 ml.), ATP (*M*/70:

TABLE V

Coupling between Formic and Glutamic Dehydrogenases

Omission from reaction mixture	No omission	Formate	DPN	α-ketoglutarate	NH_4Cl
CO_2 output *μl./hr./g.* juice (dry weight)	147	32	124	97	20

Standard system; Cotyledon juice (see the note on Tab. IV), 1.5 ml.: ormate (0.4 *M*), 0.5 ml.: DPN (5 mg./ml.), 0.9 ml.: α-ketoglutarate (0.4 *M*), 0.3 ml.: NH_4Cl (0.3 *M*), 1.3 ml.: $NaHCO_3$ (0.17 *M*), 0.5 ml. Final volume: 5 ml. Gas room; 5 vol. % CO_2 in N_2. Warburg respirometers: 30°.

TABLE VI

Coupling between Formic Dehydrogenase and Nitrate Reductase

Omission from reaction mixture	No omission	Formate	DPN	Yellow enzyme	Mb	KNO_3
CO_2 output *μl./hr./g.* juice (dry weight)	105	33	12	10	24	31

Standard system; Cotyledon juice (see Tab. IV), 1.5 ml.: formate (0.4 *M*), 0.8 ml.: phosphate buffer (*M*/10, pH 7.4), 0.5 ml.: DPN (20 mg./ml.), 0.5 ml.: old yellow enzyme (20 mg./ml.), 0.5 ml.: Mb (*M*/1,000), 0.5 ml.: KNO_3 (0.4 *M*), 0.8 ml.: $NaHCO_3$ (0.17 *M*), 0.4 ml. Final volume; 5.5 ml. Gas room; 5 vol. % CO_2 in N_2. Warburg respirometers: 30°.

0.5 ml.), DPN.2H (3 mg./ml.: 0.5 ml.), phosphate buffer (*M*/10, pH 7.4: 1 ml.), and $NaHCO_3$ (0.17 *M*: 0.5 ml.) (final volume; 3.5 ml.) could absorb not a trace of CO_2. The CO_2 fixation by coupling of formic enzyme with alcohol dehydrogenase also failed, *i.e.* a system consisting of cotyledon juice (1.5 ml.), ethyl alcohol (0.4 *M*: 0.8 ml.), ATP (*M*/70: 0.5 ml.), DPN (10 mg./ml.: 0.6 ml.), phosphate buffer (*M*/10, pH 7.4: 0.8 ml.), $NaHCO_3$ (0.17 *M*: 0.5 ml.) (made up to final volume 5.5 ml. with distilled water) was quite unable to take up CO_2. The cotyledon juice used contains considerable amount of alcohol dehydrogenase (*cf. 1*).

Effects of Metal Reagents and Cations—The effects of various metal reagents (Table VII) and cations (Table VIII) on formic enzyme were examined by the decoloration technique using purified *Preparation IV*. The results shown in Table VII are by and large in agreement with those of Davison's manometric studies, whereas the results in Table VIII do not always agree with hers.

Both KCN (*M*/500) and NaN_3 (*M*/1,000) can inhibit the anaerobic oxidation of formate completely. These reagents are known to combine with mono-, di-, and trivalent metal ions. Hypophosphite, 8-hydroxyquinoline, diethyldithiocarbamate, dimethylglyoxime, salicylaldoxime, α, α′-dipyridyl, and *o*-phenanthroline, all of which are known to be sensitive reagents for divalent metal ions, bring about a slight but

TABLE VII

Effects of Inhibitors on Formic Dehydrogenase

Inhibitors	Final conc.	Mb-reduction time (*min.*)		Inhibition $(1-\frac{T}{T'})\%$	Buffer used
		Without inhibitor (T)	With inhibitor (T′)		
Cyanide	*M*/2500	15.0	92.0	83.5	Na_2HPO_4-KH_2PO_4 (*M*/10, pH 7.4)
Cyanide	*M*/500	15.0	>300.	100.	// (//)
Azide	*M*/1000	16.5	>300.	100.	// (//)
Monoiodoacetate	*M*/1000	17.0	17.0	0	// (//)
KNO_3	*M*/500	17.0	17.0	0	// (//)
CO (light)*	100%	18.5	20.0	7.4	// (//)
CO (dark)	100%	18.5	20.8	11.0	// (//)
Hypophosphite	*M*/500	16.5	17.2	4.1	// (*M*/10, pH 5.9)
8-Hydroxyquinoline	*M*/1000	16.5	18.0	8.3	// (//)
Diethyldithiocarbamate	*M*/1000	16.5	23.8	30.7	// (*M*/10, pH 7.4)
Dimethylglyoxime	*M*/1000	16.5	24.8	33.5	NH_4OH-NH_4Cl (*M*/10, pH 5.9)
Salicylaldoxime	*M*/1000	18.5	22.1	16.1	CH_3COOH-CH_3COONa (*M*/10, pH 5.9)
α, α′-Dipyridyl	*M*/1000	19.8	23.1	14.2	Veronal-HCl (*M*/10, pH 6.8)
o-Phenanthroline	*M*/1000	19.8	22.5	11.0	// (//)
Without formate		>300			

Standard system; *Preparation IV*, 1 ml.: formate (*M*/10). 1 ml.: buffer mentioned above (*M*/10), 1 ml.: Mb (1; 10,000), 1 ml.: (DPN 2 mg.+old yellow enzyme 4 mg.)/ml., 0.5 ml.: inhibitor solution, 0.5 ml. Final volume; 5 ml. Thunberg tubes: 30°.

* Illuminated with 100 watt incandescent lamp at a distance of 15 cm.

distinct inhibiting action. CO is also able to induce an inhibition, which is scarcely alleviated by light. It seems highly probable that some metal, perhaps a divalent one, would participate in the enzyme action in question.

According to Davison (7) the KCN inhibition is of a reversible nature. In the present case, however, KCN inhibition was proved to be removed only slightly even after thorough dialysis. The following experiment may permit one to assume that under the present conditions dialysis would remove not only KCN but the very metal that is combined with the KCN added.

Preparation IV was dialysed until the *o*-phenanthroline test became negative in the outer solution of the cellophane sack. KCN (finally $M/500$) was added to the dialysate and the mixture was dialyzed again: the outer solution was concentrated, and to this $(NH_4)_2MoO_4$ was added: the appearance of yellowish brown color indicated the exudation of metal cyanide complex ions. Using the preparation "demetallized" by this KCN-treatment the effects of various metal ions were investigated. KCN ($M/500$) was added prior to the dialysis step in the procedure of the enzyme preparation (See the section of *Partial Purification of Formic Dehydrogenase*). The preparation was then dialyzed in a cellophane sack for 5 hours to drive away remaining KCN. The effects of Fe^{++}, Co^{++}, Ni^{++}, Mg^{++}, Mn^{++}, Cu^{++}, and Fe^{+++} (final concentration; $M/1,000$) on this "demetallized" enzyme preparation were examined. The results are shown in Table VIII. It is clearly shown that Fe^{++} and Co^{++} can exert a distinct activation,

TABLE VIII

Effects of Various Metal Ions on the KCN-treated Enzyme Preparation

Enzyme preparation pretreated with KCN	+	−	+	+	+	+	+	+	+	+	+
Formate	−	+	−	+	+	+	+	+	+	+	+
Metal ion added	−	$FeSO_4$	$FeSO_4$	−	$FeSO_4$	$CoCl_2$	$MgCl_2$	$MnCl_2$	$NiCl_2$	$FeCl_3$	$CuCl_2$
Mb-reduction time (*min*).	>200	>200	>200	69.0	7.5	13.0	64.0	64.0	69.0	145.0	>200

Standard system; *Preparation IV* pretreated with KCN (see the text), 1 ml.: formate ($M/10$), 1 ml.: acetate buffer ($M/5$, pH 5.9), 0.4 ml. Mb (1; 10,000), 1 ml.: (DPN 10 mg. + old yellow enzyme 20 mg./ml., 0.1 ml.: cysteine ($M/60$) as a protecting agent for the enzyme, 0.5 ml.: metal salt ($M/200$), 1 ml. Final volume; 5 ml. Thunberg tubes: 30°. Every solution in the test system was prepared using distilled water demineralized with cation exchange resin, Dowex-50.

while Cu^{++} and Fe^{+++} are strongly inhibitory. Mg^{++}, Mn^{++}, and Ni^{++} show no effect.

Distribution of Formic Acid in the Embryo Organs—Formic acid was extracted from the embryo tissues by the following procedure, and was estimated in terms of its reducing ability against ammoniacal $AgNO_3$ or $FeCl_3$ solutions.

Two hundred g. of fresh tissues to be examined were homogenized with 500 ml. of water. The homogenate was filtered through a cotton cloth by means of a hand press, and centrifuged. The supernatant was neutralized with 5 *N* NaOH and evaporated in *in vacuo* till the volume was reduced by half. It was then acidified with 20 ml. of 1 *N* H_2SO_4, and centrifuged. The precipitate was rinsed twice with 20 ml. each of 0.1 *N* H_2SO_4. The supernatant and the washing were combined. The addition of 10 g. of NaCl was followed by steam-distillation. The distillate (about 500 ml.) was alkalified with 10 ml. of 1 *N* NaOH and evaporated on a water bath. After it was almost evaporated, heating was continued for one more hour to remove aldehydes, if any. The dried material was assayed for reducing activity with ammoniacal $AgNO_3$ or $FeCl_3$. Cotyledons and seedling portions excised from 0-, 1-, 3-, and 6-day-old materials, were analyzed. A little free formate was detected only in the seedling tissues of each age, while not a bit in the cotyledon tissues.

The presence of formic acid in some ester form in cotyledon was suspected. To test this possibility, 100 g. of cotyledon meal was extracted with 200 ml. of methanol by means of a Soxhlet extractor for 3 hours. Saponification of ester that may be contained was carried out by heating the extract together with 5 g. of KOH dissolved in 10 ml. of water under a reflux condenser. After being heated for 3 hours, the solution was neutralized with CO_2-bubbling. The precipitate of K_2CO_3 was filtered off: methanol was driven away by distillation. The residual solution was acidified with H_2SO_4, and steam-distilled. The distillate was alkalified with 10 ml. of *1 N* NaOH and evaporated on a water bath. After it was almost evaporated, heating was continued for another hour, and the dried matter was assayed for the reducing activity. A positive result was obtained for cotyledons of successive ages examined (0-, 1-, 3-, and 6-day-old).

Here the possibility of aldehyde contamination was carefully excluded, since aldehydes may be present in cotyledon. It was further testified that neither aldol condensation nor the Cannizzaro reaction could be the cause of the reducing activity detected; for a mixture of 5 ml. of 36 per cent formalin, 2 ml. of 80 per cent acetaldehyde, 5 g. of KOH, and 50 ml. of methanol was heated for 3 hours just in the same way as in the saponification procedure above described, and no detectable reducing activity resulted.

DISCUSSION

Formic dehydrogenase is said to be unique in that it is inhibited by such metal reagents as cyanide and azide, and hence it has long been postulated to be a metal enzyme (*4*, *7*, *17*). The present results strongly suggest that some divalent metal ion, presumably either Fe^{++}or $Co,^{++}$

would have to do with the enzyme activity. The aerophobic nature of the enzyme shown in this study may thus be well explained as being due to the valency change by caused by aeration of the divalent metal into inactive trivalent state; cysteine that is demonstrated to be an effective remedy for the oxygen inhibition may reverse the valency from trivalent into active divalent. On the other hand, it appears that the effect of cysteine would be attributed to the possible presence of thiol group in the enzyme. It may, however not be the case because of the fact that monoidoacetate, a known thiol reagent, does not affect the enzyme (Table VII) (*7*).

In a sense, formic dehydrogenase may be grouped in the category of decarboxylase, though it apparently does not require the presence of Mg^{++} for its activity. It is remembered that Mee (*21*) who has worked on bean carboxylase has suggested that Mg^{++} should be the centre of a compound of six coordinations in which a quaternary, thiamine-pyruvate-enzyme-metal complex may be formed. It is thus tempting to assume a similar situation for the role of metal played in the formic dehydrogenase action. Both Fe^{++} and Co^{++} are known to be of six coordination number.

Standing on the thermodynamical point of view, Mathews and Vennesland (*16*) has shown that the formic dehydrogenase action in which DPN is participated can hardly be reversed in the direction of CO_2 fixation. Davison (*7*) as well as Mathews and Vennesland (*16*) could, in effect, not reverse the decarboxylation reaction. The present attempts to demonstrate the CO_2 fixation either by the mere reversal of the oxidative decarboxylation of formic dehydrogenase or by a coupling between formic and alcohol dehydrogenases proved to be equally unsuccessful even in the presence of ATP added.

Regarding the physiological role of formic dehydrogenase in sprouts, Davison (*7*) has pointed to a possibility that the anaerobic dismutation mediated by the enzyme may be of special significance in the respiration of large seeds. It is shown in this study that formic dehydrogenase can also couple with nitrate reductase with insertion of appropriate hydrogen carriers. Recently Kumada (*20*) has suggested that nitrate reductase would serve predominantly as a terminal oxidase in cotyledon as had been demonstrated for facultative anaerobionts such as *Esch. coli* (*22*, *23*, *24*). Another possible role of formic dehydrogenase may therefore be that it would take part in this acceptor respiration of cotyledon. Also in the anabolic tissues, which are aerobionts, the couplings between dehydrogenases including formic enzyme and nitrate reductase may be possible, and they would naturally be involved in the there existing vigorous nitrate assimilation mechanism (*cf.* 20).

The origin of formate in plant tissues is still an open question (*cf.* 7). Apart from this problem, it is interesting that the present study has disclosed a distinct difference as to the existing form of formic acid between the reserve tissues and the growing ones. Thus in cotyledon the bulk of formic acid seems to be stored in ester form and transported as such into the growing tissues in which the ester may be saponified to be utilized in various ways. The free acid liberated in cotyledon would rapidly be metabolized catabolically in the tissues concerned.

SUMMARY

1. Change in the formic dehydrogenase activity of various tissues of bean embryos during the course of the germination stage was investigated by the Thunberg method. In cotyledon, at the early stage of germination, the activity (on a dry weight basis) passed a high peak, and thereafter decreased rapidly as the tissues wither. The seedling part including plumules, hypocotyl, epicotyl, and radicle showed relatively weak activity throughout the germination stage, and only indistinct activity maxima were shown in respective organs at the early stage of germination.

2. The formic dehydrogenase activity was decreased by aeration. It was recovered by the addition of reducing agents, *e.g.* cysteine. For partial purification of the enzyme, anaerobic conditions or cysteine addition was found to be efficacious.

3. A respiratory system was reconstructed *in vitro* employing partially purified preparation of formic dehydrogenase, formate, DPN, flavin enzyme, and Mb. The absence of any one of these components caused considerable lowering in oxygen uptake of the system. With the insertion of appropriate hydrogen carriers, formic dehydrogenase could link with alcohol, glutamic dehydrogenases or nitrate reductase. Attempts to reverse the formate decarboxylation reaction were unsuccessful.

4. The effects of various metal reagents and of various cations were investigated. Dialysis could not remove completely the KCN inhibition. The activity of the enzyme preparation, which had been "demetallized" by KCN-treatment, was remarkably recovered by the addition

of Fe^{++} or Co^{++}.

5. Cotyledon contains formate presumably in ester form. Free formic acid was detectable only in the seedling tissues, not in cotyledon.

6. Physiological role played by formic dehydrogenase in the germinating seed and the significance of metal in the enzyme were briefly discussed.

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THE MECHANISM OF ACONITASE ACTION

III. KINETIC ANALYSIS USING DL-ISOCITRIC ACID-2-C^{14}

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In the previous reports (*1*, *2*, *3*), the author came to a conclusion that aconitase would be a single enzyme, and one enzyme and one activated complex theory was proposed. In the present communication, some additional proofs of the theory using labeled substrates will be reported.

EXPERIMENTAL

The Enzyme Preparation and the Methods of Analysis

The preparation of the rabbit liver enzyme was the same as reported previously (*1*) except it was centrifuged at $120{,}000 \times g$ for 30 minutes and the supernatant was kept. General properties of the enzyme preparation were not changed by this treatment. The preparation and the analysis of non-labeled substrates were previously reported.

The preparation of DL-*Isocitric Acid*-2-C^{14}

Usually DL-isocitric acid was prepared by hydrolysis of trichloromethylparaconic acid. It was, however, rather difficult to prepare the carbon-2 labeled compound by this method. Therefore, the compound was prepared by an entirely new process.

Ethyl Formate-C^{14}—Formic acid was prepared by the usual method from labeled barium carbonate. Its ethyl ester was prepared by esterification of the sodium salt as was done in the preparation of its methyl ester according to Melville *et al.* (*4*, *5*). The yield was about 80 per cent.

Diethyl Formyl-succinate-1-C^{14}—This compound was prepared y the method of Sugazawa (*6*). 0.6 g. of ethyl formate-C^{14} and 1.2 g. of diethyl succinate were mixed with 6 ml. of anhydrous ether. To this solution, 0.2 g. of small pieces of metallic sodium was added with cooling. The solution was maintained in the cold for 3 hours and then allowed to stand overnight at room temperature. 3 ml. of water was added in the cold and acidified with 18 *N* sulfuric acid. The water layer was extracted with small amount of ether. The combined ethereal solution was washed with small amount of water and dried. Ether and ethyl formate were distilled off and discarded. Diethyl formylsuccinate-1-C^{14} was obtained by fractional distillation under reduced pressure.

The fraction distilled from 135° to 140° at 15 mm.-Hg. was kept. The yield was 40 per cent. But, usually, it was used without the final distillation.

Crude DL-*Isocitric Acid-2-C^{14}*—To the reaction mixture (*ca.* 1 ml.) of ethyl formate and diethyl succinate, the solution of 1 gm. of sodium bisulfite in 4 ml. of water was added and vigorously stirred for 2 hours. 3 ml. of ether was added, and the mixture was discarded and the water layer was washed twice more with ether. The water layer, which contained the crystalline addition compound, was cooled with ice water and to this the cold solution of 0.8 g. of potassium cyanide in 3 ml. of water was added in several portions with vigorous stirring of 3 hours. The mixture sparated into two layers and oily upper layer consisted mainly of cyanhydrin. 3 ml. of ether was added and the reaction mixture was stirred and allowed to stand. The ethereal layer was separated and the water layer was extracted twice more with 3 ml. portions of ether. The combined ethereal extract was washed with small amount of water and ether was evaporated. 2 ml. of concentrated hydrochloric acid was added to the light yellow residue and heated at 100° for 2 hours and evaporated at this temperature. The residual mixture of oil and crystals was suspended in 3 ml. of absolute ethyl alcohol and 3 ml. of ether was added and filtered rapidly. The filtrate was evaporated. The residual oil contained DL-isocitric acid, DL-alloisocitric acid and small amount of impurities. Small amount of crystalline DL-isocitric acid was appeared after a few months. For the purification, the crude mixture was treated as follows.

Lactone of Dimethyl DL-*Isocitrate-2-C^{14}*—2 ml. of anhydrous toluene was added to the crude mixture and heated at 100° for 30 minutes and evaporated under reduced pressure. These procedures were repeated several times. After that the mixture was maintained under reduced pressure at 100° for 2 hours. By this procedure DL-isocitric acid was converted to its lactone acid. This lactone acid was dissolved in absolute ethyl alcohol and esterified with excess of ethereal diazomethane solution. The solution was evaporated and fractionally distilled under reduced pressure. The fraction distilled from 90° to 120° at 2 mm.-Hg. was kept. Small amount of crystals appeared within several hours and they were separated by filtration after 18 hours. To the filtrate 0.4 g. of the carrier lactone of dimethyl DL-isocitrate was added and distilled in the same way. The crystals separated immediately. The mixture was allowed to stand overnight in the cold and filtered. The combined crystals were recrystalized several times from anhydrous ehthyl alcohol and dried over concentrated sulfuric acid. The carrier lactone of dimethyl DL-isocitrate was prepared by the above method in larger scale or it was prepared by methylation of lactone of DL-isocitric acid prepared according to Pucher *et al.* (*7*). The labeled compound prepared without addition of the carrier and the carrier compounds prepared by the two different methods showed the same melting point at 89° and did not show the depression of the melting point when mixed each other. The elementary analysis showed they were of the same composition; for the first preparation, the result was as follows;

Calcd. for $C_8H_{10}O_6$ (202): C 47.50, H 4.99
Found : C 47.57, H 5.05.

The yield of lactone of dimethyl DL-isocitrate-2-C^{14} from diethyl formyl-succinate-1-C^{14}

was about 7 per cent of the theoretical amount. The low yield might be due to the formation of allo-compound and the repeated recrystallization.

Solution of Sodium DL-*Isocitrate-2-C^{14}*—Lactone of dimethyl DL-isocitrate-2-C^{14} was hydrolyzed by warming with excess of 0.1 *N* sodium hydroxide and after that it was neutralized to pH 7.4 with 0.1 *N* hydrochloric acid. The concentration of neutralized solution was represented as that of *d*-isomer.

The Degradation Procedures

Lactone of dimethyl DL-isocitrate was directly oxidized according to the Pregl's dry combustion method. Citric acid and cis-aconitic acid were converted to pentabromoacetone and oxalic acid respectively. The general outline of the proposed procedure was as follows: After stopping the enzyme reaction by the addition of trichloroacetic acid, small aliquot of sample was removed for the determinations of the contents of the tricarboxylic acids. To the remaing solution, suitable amount of the carrier citric acid (50 to 100 μM) and *cis*-aconic acid (200 μM) were added and centrifuged. The supernatant was neutralized with 4 *N* sodium hydroxide and the tricarboxylic acids were precipitated as silver salts by the addition of 2 *N* silver nitrate. The silver salts were dissolved in 10 per cent solution of ammonium hydroxide and silver was removed with 2 *N* potassium bromide and centrifuged off. The supernatant was cooled with ice water and was carefully oxidized by dropwise addition, with stirring, of cold 0.2 *M* potassium permanganate (4/3 M of permanganate per 1 M of *cis*-aconitate). After one hour, residual permanganate was decomposed with 1 per cent solution of hydrogen peroxide and centrifuged. The supernatant was acidified with glacial acetic acid and oxalic acid was precipitated by the addition of 1 *M* calcium acetate. The precipitated calcium oxalate was dissolved in 2 *N* hydrochloric acid and centrifuged. Glacial acetic acid was added to the supernatant and after that the solution was adjusted to pH 4 by the addition of 2 *N* sodium hydroxide with vigorous stirring. In this way, precipitation and solution of calcium oxalate were repeated three times and the final precipitate was washed with boiling glacial acetic acid and then with water. Calcium oxalate was dissolved in 1 *N* hydrochloric acid and oxidized with 0.2 *M* potassium permanganate. The supernatant of the first calcium oxalate precipitation was acidified with 18 *N* sulfuric acid and citric acid was converted to pentabromoacetone by oxidation with potassium permangante in the presence of potassium bromide and bromine solution by the usual way. Pentabromoacetone was purified by sublimation under reduced pressure and oxidized according to the Pregl's dry combustion method. Carbon dioxide was always converted to barium carbonate.

Determination of Radio Activity

The measurement of radio activity was done by an end window Geiger-Müller counter and corrected for self-adsorption (*5*). The specific activity of lactone of dimethyl DL-isocitrate was 103,000 c.p.m./mM. Assuming this compound to be 100 per cent labeled, the specific activity of pentabromoacetone from 100 per cent labeled citric acid and that of oxalic acid from 100 per cent labeled *cis*-aconitic acid were calculated as 275,00 and 412,000c.p.m./mM, respectively, and those of carbon-2s of 100 per cent labeled tricarboxylic acids were calculated 824,000 c.p.m./milli atom equivalent. These calculasted values were confirmed by experiments and they coincided with the experimental values within the error of estimation. The amounts of the labeled compounds were calculated from the total amounts of acids and their respective specific activities. The amounts of non-labeled compounds were calculated as the difference between the total amounts and the amounts of labeled compound.

RESULTS

Inhibition of Non-labeled Citrate Formation from Cis-Aconitate by DL-*Isocitrate*-2-C^{14}

If two substrates were mixed and one of them was labeled the origins of the products could be made clear by tracing radio activity. The constant amount of DL-

TABLE I

Inhibition of the Reaction of Citrate Formation from Cis-aconitate by DL-*Isocitrate*-2-C^{14}

Starting from			After 30 minutes				
DL-Isocitrate-2-C^{14}	*Cis*-aconitate	1/A	Citrate				1/*v*
Total μM	Total μM		Total μM	Specific Activity *c.p.m.*	Labeled μM	Non-Labeled μM	
40	15	666	0.80	320,000	0.32	0.48	2.1
40	10	1000	0.77	400,000	0.37	0.40	2.5
40	7.5	1333	0.67	470,000	0·38	0.29	3.4
0	15	666	1.20*			1.20*	0.83
0	7.5	1333	1.14*			1.14*	0.88

The amounts of acids are expressed in μM/10 ml. A is the concentration of *cis*-aconitate in mole/lit. Specific activities are those of carbon-2 before addition of the carrier in c.p.m./milli atom equivalent. *v* is the rate of formation of non-labeled citrate in μM/10ml./30 minutes. The values with the asterisk were determined after 10 minutes and multiplied by three.

3 ml. of DL-isocitrate-2-C^{14} solution, 2 ml. of *cis*-aconitate solution and 5 ml. of the enzyme solution in 0.02 *M* phosphate buffer of pH 7.4 were incubated at 25°. After 30 minutes, 2 ml. portions were used for the determination of total citrate and 8 ml. portions were used for the determination of the specific activities after addition of 50 μM of carrier citrate.

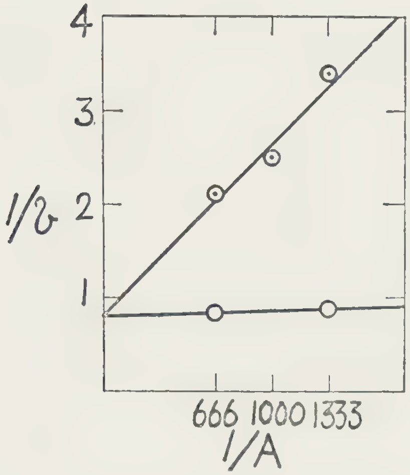

FIG. 1. Inhibition of the reaction of citrate formation from *cis*-aconitate by DL-isocitrate-2-C^{14}.

This figure was obtained from the experimental results presented in Table I.

isocitrate-2-C^{14} and varying amounts of *cis*-aconitate were mixed and the rate of formation of non-labeled citrate from *cis*-aconitate was determined. The experimental results, as presented in Table I and Fig. 1, showed that this reaction was inhibited by DL-isocitrate-2-C^{14}. Similarly, the reaction of formation of lebeled *cis*-aconitate from DL-isocitrate-2-C^{14} was inhibited by non-labeled citrate.

Change of Specific Activities of Citrate and cis-Aconitate When the Reaction was Started from the Mixture of Non-labeled cis-Aconitate and DL-*Isocitrate*-2-C^{14}

If *cis*-aconitate was an essential intermediate of the reaction of formation of citrate from isocitrate, the specific activity of *cis*-aconitate, after a certain time, would be greater than that of citrate when labeled isocitrate and *cis*-aconitate were mixed. But if *cis*-aconitate was not an essential intermediate, the reverse result would be expected.

Fig. 2 A shows the changes in the amounts of the three compounds which were derived from a mixture of DL-isocitrate-2-C^{14} and *cis*-aconitate. Because they were mixed in the mole ratio of 3.7 : 1, the amount of *cis*-aconitate did not change for fairly long period while citrate increased continuously and isocitrate decrease (Fig. 2 of Ref. (*2*)). At every five minutes, specific activities of carbon-2s of citrate and *cis*-aconitate were determined and the results were presented in Fig. 2 B. Relative values of specific activities were calculated assuming that of carbon-2 of DL-isocitrate as 100 per cent and

Fig. 2 A Fig. 2 B

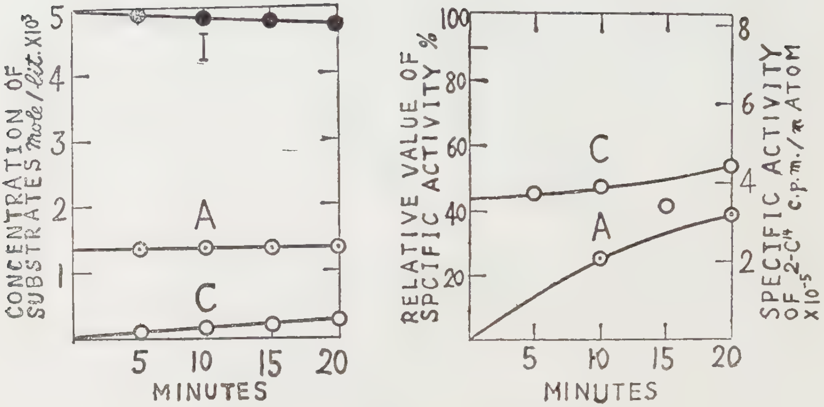

Fig. 2 A and B. Change of amounts and specific activities of citrate and *cis*-aconitate.

The solution of 250 μM of DL-isocitrate-2-C^{14} and 68 μM of *cis*-aconitate in 30 ml. of water and 20 ml. solution of the enzyme in 0.02 *M* phosphate buffer of pH 7.4 were incubated at 25°. Four 2 ml. portions were used for the determination of concentration of substrates and four 10 ml. portions were used for the determination of specific activity after addition of 100 μM of citrate and 200 μM of *cis*-aconitate. The specific activity in the ordinate is those of carbon-2 before addition of carrier in c.p.m./milli atom equiualent. On the relative value of specific activity, read in the text.

they were also presented in the same figure.

In the experiments shown in Fig. 3, DL-isocitrate-2-C^{14} and *cis*-aconitate were mixed in varying ratios, which were shown in the abscissa, and the ratio of the amount of labeled to non-labeled citrate were determined and pressented in the ordinate. The points obtained are on a straight line.

DISCUSSION

The inhibition experiments proved that the three substrates combined competitively and reversibly with a same reaction center of the enzyme molecule. Moreover, the dissociation constants of the inhibitors were nothing but their respective Michaelis constants.

The last two experiments could not be explained considering *cis*-aconitate as an essential intermediate of the reaction of formation of

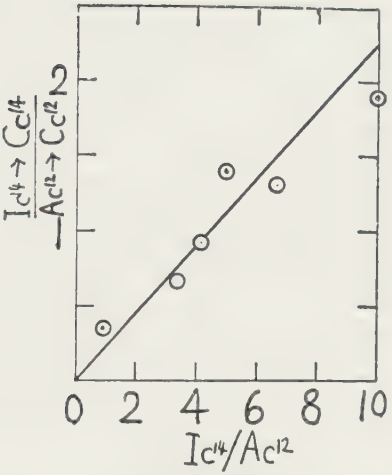

FIG. 3. The relation between the ratio of the rates of formation of labeled citrate to non-labeled citrate and the ratio of the amounts of DL-isocitrate-2-C^{14} to *cis*-aconitate.

The circles were obtained from the reactions presented in Table I. The dotted circles were obtained from the analogous experiments using 40, 8, and 4 μM/10 ml. of *cis*-aconitate, respectively.

citrate from isocitrate. On the other hand they could be clearly explained by the one enzyme and one activated complex theory. Concerning the ratios of the rate of activation of the substrates, the following relation is expected from the theory.

Ratios of the rate of activation of the substrates are

1 (C) : 45 (A) : 10 (I) Relation A of Ref. (*2*)

(Abbreviations are the same as those in the previous paper (*1*)).

In case *cis*-aconitate and DL-isocitrate-2-C^{14} were mixed, the ratio of the rate of activation of them was 45 (A) : 10 (I). This value would be directly concerned with the specific activity of citrate formed. Therefore, when (A) : (I) was 1 : 3.7 as in the case of the experiment presented in Fig. 2A and B, the relative value of the specific activity of carbon-2 of citrate formed in the initial stage was expected to be 100 per cent $\times \frac{37}{45+37} = 45$ per cent, and the value obtained was about 43 per cent. The values of *cis*-aconitate were slightly larger than expected, it may probably be due to the error of estimation, but they were always smaller than the corresponding values of citrate. Fig. 3 shows the first order relation of the ratio of the concentration of substrates and the ratio of the rate of formation of labeled and non-labeled citrate from them. The line is the expected one from the theory and the experimental results satisfy the theory.

In using isotopic techniques for the rate analysis, it must not be forgotten to consider the following experimental facts. In the experiment of their "malic" enzyme preparation, Kaufman *et al.* (*8*) concluded that pyruvate is not a free intermediate in the over-all reaction of the formation of L-lactate and carbon dioxide from L-malate and suggested that "malic" enzyme and lactic dehydrogenase may be bound in a complex such that intermediary pyruvate is reduced to lactate faster than it pools with the pyruvate in solution. In the case of aconitase, however, it could be possible to neglect such possibility from the experimental results on the kinetic analysis presented before and in this paper.

SUMMARY

DL-Isocitric acid-2-C^{14} was synthesized and the methods of degradation of citric acid-2-C^{14} and *cis*-aconitic acid-2-C^{14} were presented.

DL-Isocitrate-2-C^{14} competitively inhibited the reaction of citrate formation from *cis*-aconitate.

When the reaction was started from the mixture of DL-isocitrate-2-C^{14} and *cis*-aconitate, the specific activity of citrate formed after a certain period was greater than that of *cis*-aconitate presented at the same time and the ratio of the rate of formation of labeled to non-labeled citrate was the first order function of the ratio of the amount of DL-isocitrate-2-C^{14} to *cis*-aconitate.

These results were easily explained by the author's theory.

The author is indebted to Dr. M. Izawa for the determination of radio activity.

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ISOLATION OF CRYSTALLINE TAKA-AMYLASE A FROM "TAKADIASTASE SANKYO"

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In 1951 Ed. H. Fischer *et al.* (*1*) reported on the isolation of crystalline taka-amylase from "Clarase 900" by fractional precipitation with ammonium sulfate and acetone. In the authors' laboratory Hayashi (*2*) has previously reported on the fact that taka-amylase is almost completely precipitated by rivanol (2-ethoxy-6,9-diamino-acridinium lactate) from salt free aqueous solutions. By removing rivanol from the taka-amylase-rivanol symplex he could obtain a highly purified taka-amylase preparation. The authors (*3*) have been able to obtain crystalline taka-amylase from a synthetic culture medium of *Aspergillus oryzae* by a combined procedure of the rivanol treatment and fractional precipitation by acetone.

The present paper deals with the isolation of crystalline taka-amylase from "Takadiastase Sankyo" by a relatively simple method and in a good yield. The purfication and crystallization procedures are summarized in Table I.

Recently, Okazaki (*4*) reported on the presence of a new saccharogenic enzyme of lower molecular weight and higher solubility in "Takadiastase Sankyo," and therefore, the crystalline tata-amylase obtained by the authors should be named "Taka-amylase A."

For the crystallization of taka-amylase A the presence of calcium ion is necessary and consequently crystalline taka-amylase A contains a small quantity of calcium and seems to be calcium salt of the enzyme.

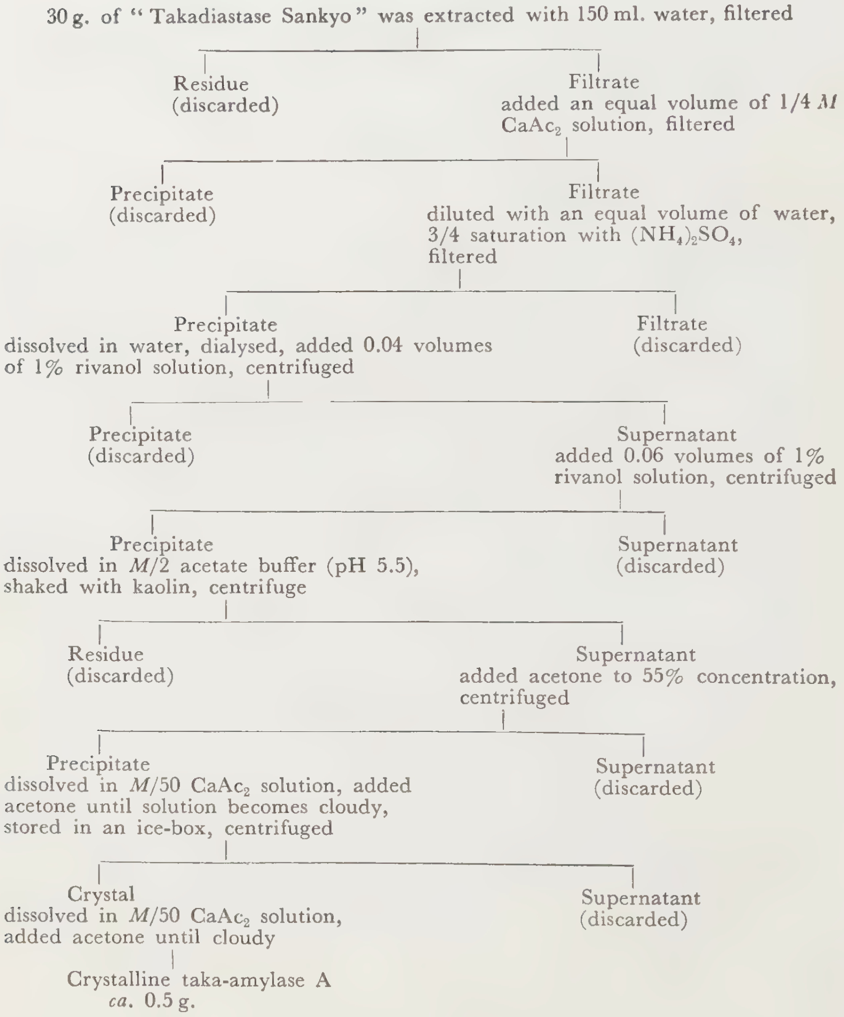
The yield of crystalline taka-amylase A is about 0.5 g. from 30 g. of "Takadiastase Sanko," which is approximately 30 per cent in enzyme units.

EXPERIMENTAL

Amylase Activity—A modification of Willstätter-Schudel's method was employed for the determination of amylase activity. In an 100 ml. glass-stoppered

TABLE I

Scheme of Purification Procedures of Taka-amylase A



30 g. of "Takadiastase Sankyo" was extracted with 150 ml. water, filtered

Residue (discarded)

Filtrate added an equal volume of 1/4 M $CaAc_2$ solution, filtered

Precipitate (discarded)

Filtrate diluted with an equal volume of water, 3/4 saturation with $(NH_4)_2SO_4$, filtered

Precipitate dissolved in water, dialysed, added 0.04 volumes of 1% rivanol solution, centrifuged

Filtrate (discarded)

Precipitate (discarded)

Supernatant added 0.06 volumes of 1% rivanol solution, centrifuged

Precipitate dissolved in $M/2$ acetate buffer (pH 5.5), shaked with kaolin, centrifuge

Supernatant (discarded)

Residue (discarded)

Supernatant added acetone to 55% concentration, centrifuged

Precipitate dissolved in $M/50$ $CaAc_2$ solution, added acetone until solution becomes cloudy, stored in an ice-box, centrifuged

Supernatant (discarded)

Crystal dissolved in $M/50$ $CaAc_2$ solution, added acetone until cloudy

Supernatant (discarded)

Crystalline taka-amylase A *ca.* 0.5 g.

flask 20 ml. of substrate (prepared by mixing 2 parts of 1.65 per cent soluble starch solution and 1 part of 0.1 M pH 5.3 acetate buffer) was placed. This solution was brought to 37° in a thermostat bath, and 2 ml. of properly diluted amylase solution was added. After 10 minutes, 10 ml. of 0.2 N sodium hydroxide solution was added to stop the digestion followed by the addition of 10 ml. of 0.1 N iodine solution. After being kept at room temperature for 15 minutes, an excess of dilute sulfuric acid was added and titrated with 0.05 N thiosulfate. A control was run adding the enzyme solution only after 0.2 N NaOH solution had been added. Each ml. of 0.1 N iodine is equivalent to 17.15 mg. of maltose.

The authors used a new unit which is defined as the amount of enzyme which produces 17.15 mg. of maltose in 10 minutes under the above mentioned conditions. Specific activity is expressed as amylase unit per mg. protein nitrogen, A.U./P.N. Protein nitrogen was estimated by usual micro Kjeldahl method.

Extraction of Enzyme—Thirty g. of "Takadiastase Sankyo" were stirred well with 150 ml. of cold water, and after 3 hours the solution was filtered by a large Büchner funnel. The residue was washed with 10 ml. of water. To the combined filtrates and washings, equal volume of $M/4$ solution of calcium acetate was added under stirring, while the pH was adjusted to 6.8–7.0 by adding 5 N NaOH solution. The precipitate formed was removed by filtration with the aid of celite. The filtrate (330 ml.) was diluted with equal volume of water and brought to 3/4 saturation with ammonium sulfate by adding about 350 g. of solid ammonium sulfate under continuous stirring. The precipitate was collected on a Büchner funnel, washed with a small volume of 3/4 saturated ammonium sulfate solution and dissolved in cold water. The solution was dialysed in a collodion bag against running water 2 days and then against distilled water overnight. The main part of amylase activity in Takadiastase was found in this solution.

Purification by Rivanol—To the dialysed solution described above (*ca.* 320 ml.) 12 ml. of 1 per cent aqueous rivanol solution was added and the dark yellow precipitate thereby formed was removed by centrifugation. To the supernatant, 1 per cent rivanol solution was again added until yellow precipitate formed increased no more. By this treatment, only taka-amylase A was precipitated and separated almost completely from other proteins. The precipitate collected by centrifugation was dissolved in 50 ml. of 0.5 M acetate buffer (pH 5.5) and decolorized by shaking with 10 g. of kaolin (or Japanese acid clay). The taka-amylase solution obtained by filtration from the kaolin was clear and almost colorless.

Precipitation with Acetone and Crystallization—From the colorless solution obtained as above, taka-amylase A was precipitated by adding acetone to 55 per cent concentration in ice cold. The precipitate obtained by centrifugation was dissolved in a small volume of ice cold 0.02 M solution of calcium acetate. After the insoluble substance was removed by centrifugation, acetone was added gradually until the solution became cloudy and it was stored in an ice box, where taka-amylase A crystallized out in microscopic cubes (Fig. 1). The final concentration of acetone was brought to about 45 per cent in order to complete the crystallization.

Recrystallization—The crude crystals were dissolved in a small quantity of 0.02 M solution of calcium acetate and recrystallized as above in ice cold by adding acetone.

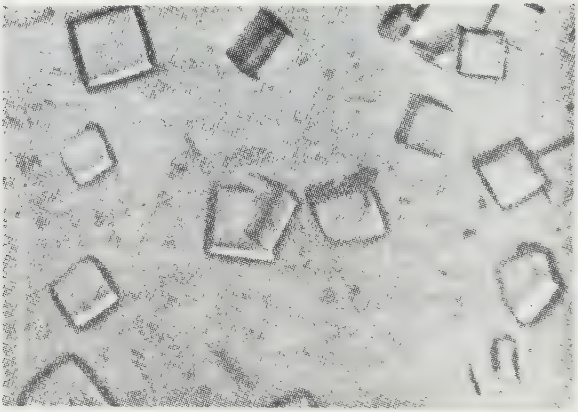
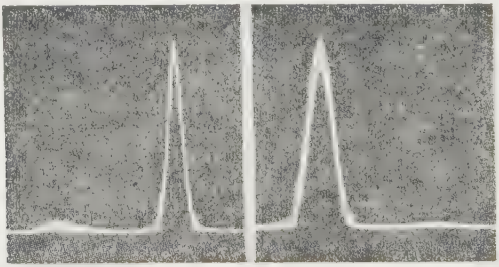


FIG. 1. Crystalline taka-amylase A (×1000)

The amylase activity and purity of taka-amylase A in each step of purification is shown in Table II.

Properties of Taka-amylase A

Crystalline taka-amylase A is electrophoretically homogeneous, as fas as measured at pH 4.1, 5.0, 5.6 and 8.0. Fig. 2 shows an electrophoretic diagram obtained with one of the preparations after leaving 80 minutes in 0.2 *N* acetate buffer at pH 5.0. The isoelectric point was estimated to be around pH 3.7 by electrophoresis. This value is lower than that reported by Fischer *et al.* (*1*)



Ascending boundary Descending boundary

FIG. 2. Electrophoretic diagram of taka-amylase A: acetate buffer pH 5.0, $\mu=0.2$, 4860 seconds. mobility 5.12×10^{-5} cm.^{2}/sec./volt.

The results of elemental analysis of taka-amylase A (dried over P_2O_5 at 56° ***in vacuo***) are as follows.

TABLE II

Amylase Activity and Purity of Taka-amylase A in Each Purification Step

Procedure	Total amylase activity	Yield	Specific activity
	A.U.	%	*A.U./P.N.*
Extraction with water	50,000	100	—
$CaAc_2$ treatment	48,500	97	—
Salting out with $(NH_4)_2SO_4$	33,000	66	137
Rivanol treatment	24,000	48	203
Precipitation with acetone	20,600	41	205
First crystallization	16,400	33	208
Recrystallization	13,700	28	206

C	H	N	S	Ash	P
48.10	7.47	14.98	0.68	0.73(Ca)	— (by the authors)
47.22	7.05	12.08	1.11	1.93	0.03(by Hanrahan and Caldwell (*5*))
—	—	12.9	—	—	0.03(by Fischer and Montmollin (*6*))

The authors' result for nitrogen content of taka-amylase is distinctly higher than that described by Hanrahan *et al.* (*5*), and Fischer *et al.* (*6*).

This enzyme has no maltase and phosphatase activities, but has a weak digestive activity for horse serum and haemoglobin at pH 3 to 4.

The crystalline enzyme has all properties of protein, gives positive Millon's and biuret reactions and also positive Molisch reaction as reported by Hanrahan and Caldwell (*5*).

SUMMARY

Crystalline taka-amylase A was obtained in a good yield from "Takadiastase Sankyo" using rivanol (2-ethoxy-6,9-diamino-acridinium lactate) as a specific precipitation reagent. The crystals seems to be calcium salt of the enzyme protein. The protein is electrophoretically pure.

The authors wish to express their gratitudes to Sankyo Co., Ltd. for their kind supply of "Takadiastase Sankyo" and to Mr. Ohta for his technical assistance in electrophoresis.

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A NEW METHOD FOR MICRODETERMINATION OF AMYLASE ACTIVITY BY THE USE OF AMYLOSE AS THE SUBSTRATE

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Regardless of the source and the purity of an amylase preparation, amylase action is characterized by the simultaneous changes of the following properties of the substrate: (a) decrease of viscosity, (b) increase of reducing power, (c) change in iodine-color reaction, (d) change in optical rotatory power, and (e) decrease in the turbidity of glycogen solution (*1*). The methods described in the literature for the determination of amylase activity are numerous (*1, 2*). Most of the authors use one of the above five phenomena, especially (a), (b), or (c). Amylase activity is expressed as (a) liquefying power, (b) saccharifying power, or (c) dextrinizing power, respectively, according to the method used.

It is known that numerical values of amylase power obtained by a given method differ from those obtained by other methods no matter how closely brought the conditions (temperature, pH, time, volumes of reagents used), depending upon the nature of starch or soluble starch used as the substrate.

Since Meyer (*3*) discovered in 1940 that most starches are composed of two different types of polysaccharides, namely amylose, linear component, and amylopectin, branched component, various methods have been developed for the preparation of these two components (*4*). Recently, we have been able to find a relatively simple method whereby a fairly large amount of the pure amylose can be obtained (*5*). In the meantime, some investigators have clarified to a certain extent the natures of the action of several amylases upon amylose. Therefore, it would be better to use amylose as the substrate, instead of starch and soluble starch which are composed of a mixture of amylose and amylopectin, in order to obtain more accurate results in the determination of activity of amylase action.[2]

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The author examined several methods to find a procedure which would give rapid, easy, and accurate dextrinizing and saccharifying powers of amylase. Andersch (*8*) used potato β-amylose as the substrate to estimate the activity of serum amylase by the saccharimetric method. Hobson *et al.* (*9*) and Kung *et al.* (*6*) also used amylose as the substrate for the determination of α-amylase activities. The present paper deals with the methods employed in the colorimetric microdetermination of dextrinizing power (D.P.) and saccharifying power (S.P.) of amylase by the use of amylose as the substrate. For D.P. determination, decrease in the amount of the substrate was measured by the change of blue values of amylose-iodine complexes according to a modified method of that described by McCready *et al.* (*10*). For S.P. determination, an amount of reducing substrates formed in the course of amylase action was determined by using a combination of Somogyi's copper reagent (*11*) and Nelson's arsenomolybdate reagent (*12*).

EXPERIMENTAL

Amyloses

a) *Preparation of Amylose*—Amyloses were prepared from potato, corn, and sweet potato starches by the authors' "precipitant-enzyme method" (*5*) and from potato starch by Schoch's butanol precipitating method (*13*)[3]. Amylose-complexes crystallzed three times were collected and dried according to the usual method. The powdered dry amyloses contained about 2 per cent moisture and were stored in desiccator over calcium chloride. In the preparation of amylose solutions weights of amylose were not corrected for moisture.

b) *Preparation of* 0.2 *Per Cent Amylose Solution*—A weighed amount of amylose (approx. 200 mg.) was dissolved in 4 ml. of 1 *N* solution of NaOH by allowing to stand overnight in the refrigerator (*14*). After diluting it to about 80 ml. with water in a 100 ml. volumetric flask, the solution was made just acid to phenolphthalein with 1 *N* solution of AcOH and diluted to the mark with water. The solution was kept in the constant temperature water bath (37±0.1°). After a 6 hour standing the solution did not show any appreciable retrogradation which would give influences upon amylase action. The solution was prepared daily.

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[2] Literatures on these problems are presented by Kung *et al.* (*6*) and Hopkins *et al.* (*7*) in their recent papers.

[3] The following abbreviation will be used for these amyloses in this paper; P.E.-amylose and B-amylose.

Amylases

a) *Crystalline Taka Amylase* [*α-Type*][4]—Taka amylase [α-type] was obtained in crystalline form from Taka-diastase powder according to the method described by Akabori *et al.* (*16*). Crystals crystallized twice were dissolved in water and the solution was stored in the refrigerator as the stock solution of crystalline Taka amylase [α-type]. The stock solution contained 2.86 mg. of protein nitrogen per ml. For the determination of D.P. and S.P. proposed methods, 1 ml. of the stock solution was diluted from 25×10^4 to 100×10^4 times and from 6×10^4 to 25×10^4 times, respectively.

b) *Crystalline Bacterial Amylase* [*α-Type*][4]—The crystals of bacterial amyloclastic amylase [α-type] purified twice by recrystallization were prepared from culture medium of *Bacillus subtilis* by Hagihara's method (*17*). The sample was kindly furnished to the author by Mr. Hagihara. The crystals were suspended in a small amount of a solution of $M/100$ calcium acetate and dissolved by the dropwise addition of $N/10$ solution of NH_4OH adjusting pH of the solution to about 10. This solution contained 0.37 mg. of protein nitrogen per ml. and was stored in the refrigerator as the stock solution of crystalline bacterial amylase [α-type]. The solution of this amylase should be diluted with $M/100$–$M/200$ solution of calcium acetate, otherwise amylase will be inactivated by dilution. For the determination of D.P. and S.P. proposed methods, each ml. of this stock solution was diluted from 5×10^4 to 25×10^4 times and from 0.5×10^4 to 3×10^4 times, respectively.

c) *Crystalline Sweet Potato Amylase* [*β-Type*][4]—Crystalline sweet potato amylase [β-type] was prepared by the method of Balls *et al.* (*18*) and kindly sent to the author by Baba. The crystals separated from a 0.5 saturated solution of ammonium sulfate were collected by centrifugation and dissolved in a small amount of water. The solution contained 0.36 mg. of protein nitrogen per ml. and was stored in the refrigerator as the stock solution of crystalline sweet potato amylase [β-type]. For the determination of D.P. and S.P. proposed methods, each ml. of this stock solution was diluted from 1000 to 5000 times and from 1000 to 2500 times, respectively.

d) *Purified Malt Amylase* [*α-Type*][4]—Barley malt amylase [α-type] was purified by a modified method of crystallization procedure used by Schwimmer *et al.* (*19*). The enzyme was extracted from powdered brewing malt by adding of water 10 times the quantity of the malt and by incubating the mixture at 30° for 3 hours with occasional shaking in the presence of toluene. To the filtered extract was added 1 M solution of calcium acetate at pH 7 so that the concentration becomes $M/10$ with respect to calcium acetate added and the precipitate formed was removed by filtration. The filtrate was heated at 70° for 15 minutes at pH 7.0 for the purpose of destroying β-type amylase and other enzymes except α-type amylase and again filtered. The filtrate was concentrated under vacuo at temperature not exceeding 40° to about 1/5 of the original volume, and the residual was dialyzed in cellophane against running tap water for a day. Crystalline ammonium sulfate was added to the dialyzed solution to 0.5

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[4] This nomenclature of amylases was proposed by the authors (*15*).

saturation, the precipitate salted out was collected and dissolved in cold water. The process of salting out was repeated once more and the precipitate collected was dissolved in a small amount of cold water. After dialysis through cellophane, with addition of toluenen, against running tap water for 2 days, the enzyme solution was dialyzed further for several more days against a $M/100$ solution of calcium acetate changing with fresh solution from time to time. The dialyzed solution was stored in the refrigerator as the stock solution of purified malt amylase [α-type]. Dextrinizing power of this solution measured by the modified method of Ehrnst *et al.* (*20*) over that of Wohlgemuth was found to be D=600 per ml., and saccharifying power measured by the method modified over that of Willstätter-Schuldel (*21*) was A.E. (Amylasen Einheit)=0.1 per ml. For the determination of D.P. and S.P. proposed methods, each ml. of the stock solution was diluted from 1250 to 5000 times and from 200 to 1000 times, respectively.

e) *Extract from Powder of Germinated Rice Grain* (*22*)—Germinated rice grains (germinated at 30° for 7–10 days in dark, dried at 55–60° for 15 hours, powdered, and passed through a mesh of 0.5 mm.) were extracted with water ten times of its weight v/w) at 30° for 3 hours with occasional shaking in the presence of toluene. The filtered extract was used as the enzyme solution. Amylase activities of this solution were $D_{66}^{37°}=25-35$ and A.E.$=0.01-0.02$ per ml. For the determination of D.P. and S.P. proposed methods, each ml. of the stock solution was diluted from 100 to 200 times. It was necessary to run blank tests to obtain reducting values for the enzyme solutions in the case of the saccharimetric methods.

Determination of Protein Nitrogen

Amylase proteins were precipitated with trichlorocacetic acid (final concentration 7 per cent) from 1 ml. of the stock solutions of amylases filtered, and washed with 7 per cent trichloroacetic acid solution. Nitrogen content of the washed trichloroacetic acid precipitate was determined by the usual micro-Kjeldahl method.

Measurement of Dextrinizing Power (*D.P.*)

a) *Reagent A—A Mixture of a* 0.2 *Per Cent Solution of Iodine and a* 2 *Per Cent Solution of Potassium Iodide*—This was prepared daily from the stock solution containing 1 per cent iodine and 10 per cent potassium iodide by diluting with water.

b) *Procedure*—The total volume of a mixture of 1 ml. of 0.5 M acetate buffer5) and the enzyme solution was made up to 2.5 ml. with water in Tube A. Tube B, a control, contained the same total volume but substituting water for the enzyme solution. Both tubes were kept in a constant temperature water bath at 37 ±0.1° for about 10 minutes and to each was added 2.5 ml. of 0.2 per cent amylose

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5) In our experiments, buffer of pH 6.0 was used for bacterial amylase and of pH 5.0 for other amylases.

solution. At the end of a 30 minutes period after the addition of the amylose solution, 5 ml. of 1 N solution of AcOH added to each of the two tubes. The tubes were shaken. Contents of the tubes were transferred quantitatively into 250 ml. volumetric flaks, diluted with about 200 ml. of water, to these was added each 5 ml. of reagent A, and the mixtures were diluted to the mark with water. Colorimetric readings on the logarithmic scale (optical densities) were taken on a photoelectric colorimeter[6] fitted with a S_{70} filter[7] and a 10 mm. glass cell. The measuring instrument was set to zero against water.

c) *Calculation of Amylase Activity*—Dextrinizing power (D.P.) was calculated according to the following equation.

$$\text{D.P.}=\frac{D_0-D}{D_0}\times 100\div 10$$

where D = optical density found for Tube A
D_0 = optical density found for Tube B

Consequently amylase unit (1 D.P.) is defined as the amount of amylase which will produce 10 per cent fall in the intensity of blue color of amylose-iodine complexes under the conditions mentioned above.

Measurement of Saccharifying Power (S.P.)

a) *Reagent A (Somogyi's Copper-Phosphate-Tartrate Reagent)*—This was prepared according to the formula described by Somogyi (*11a*).

b) *Reagent B (Somogyi's New Copper-Carbonate-Tartrate Reagent for Colorimetry)*—This was prepared according to the formula described by Somogyi (*11b*).

c) *Reagent C (Nelson's Arsenomolybdate Reagent)*—This was prepared according to the formula described by Nelson (*12*).

d) *Reagent D. Standard Solution of* 0.02 *Per Cent Glucose*—Four hundred mg. of glucose was dissolved in 200 ml. of *p*-hydroxybenzoate saturated aqueous solution. The solution was stored in the refrigerator as the stock solution. Five ml. of the stock solution were diluted to 50 ml. with water daily. Two ml. and 1 ml. of the diluted solution were used for standardization of glucose at 0.4 mg. and 0.2 mg., respectively.

e) *Procedure*—The total volume of a mixture of 0.5 M acetate buffer (0.5 ml.)[5] and the enzyme solution was made up to 1.5 ml. with water in Tube A. The tube was kept in a constant temperature water bath at 37±0.1° for about 10 minutes and added 1.5 ml. of 0.2 per cent amylose solution. At the end of a 30 minute period after the addition of the amylose solution, the reaction mixture in the tube and 3 ml. of reagent A or B[8] were mixed and the minutes was heated for 30 minutes in a boiling-water bath. To the cooled solution were added 3 ml. of reagent C[9], whereby the cuprous oxide was brought into solution by mild agitation. After 20 minutes the blue solution was transferred quantiatively into a 100 ml. volumetric flask, diluted to the mark with water, and mixed. Readings of optical densities were made with a photoelectric colorimeter[6] fitted with a S-50 filter[10] and a 20 mm. glass cell. The measuring instrument was set to zero against a reagent blank which had been prepared according to the same procedure, except that amylose and enzyme solutions were absent. When the enzyme solution itself had reducing power, readings of optical densities were made on an enzyme blank which had also been prepared according to the same procedure except omitting the addition of amylose.

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[6] AKA-type of Kotaki-Seisakusho.

[7] Gelatin filter transmitting light of wave length 700 mμ.

[8] Reagent B is superior to reagent A owing to its stability during storage and stability of color developed. Therefore reagent B is used in the author's laboratory now. However almost all experiments in this paper were done with reagent A.

A standard calibration curve for aqueous glucose solution under the standard conditions was prepared at each estimation. From the curve mg. of glucose equivalent to a unit optical density was obtained.

f) *Caluculation of Amylase Activity*—Saccharifying power (S.P.) was calculated according to the following equation.

$$\text{S.P.}=\text{G}\times(\text{S}-\text{S}_0)\times\frac{1}{0.1}$$

where G = mg. of glucose equivalent to one optical density

S = optical density for Tube A

S_0 = optical density observed for the enzyme blank

One amylase unit (1 S.P.) is defined as the amount of amylase which will produce reducing substances equivalent to 0.1 mg. of glucose under the conditions mentioned above.

RESULTS

Relation of the Concentration of Amylose to the Color Intensity of Amylose-iodine Complex—The relationship between the concentration of potato P.E.- and B-amyloses and the color intensities of amylose-iodine complexes as judged by blue values is shown in Fig. 1. The color densities are excellent in accordance with Beer's law at a range between 0.5 to 5.0 mg. of amylose per 250 ml. Therefore it will be possible to measure the amount of decrease of the substrate amylose by the action of amylase through observing change of blue values under these conditions.

The solution contained 5 ml. of 1 *N* solution of AcOH, 5 ml. of a mixture of 0.2 per cent solution of iodine and 2 per cent solution of KI, 1 ml. of 0.5 *M* acetate buffer of pH 5.0 and appropriate amount, 0.25–2.5 ml., of 0.2 per cent amylose solution per 250 ml. Colorimetric readings were made with a photoeletric color meter[6] fitted with a a S-70 filter[7] and 10 mm.-glass cell. The measuring instrument was set to zero against water. Optical densities were not corrected for moisture of amylose.

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[9] The amount of reagent C to be added need not exceed 2 ml. in the case of reagent B.

[10] Gelatin filter transmitting light of wave lengh of 500 mμ.

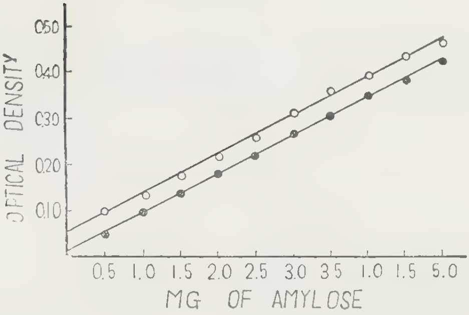

FIG. 1. Relation of the concentration of amylose to the color intensity of amylose-iodine complex.

○—○ Potato P.E.-amylose: ●—● potato B-amylose.

(D.P.): The data given in Fig. 2 illustrate the fact that D.P. values measured by the proposed method are strictly proportional to the amount of enzyme present in purified malt amylase [α-type], crystalline Taka amylase [α-type], and crystalline sweet potato amylase [β-type], provided D.P. values measured were not more than 5 units

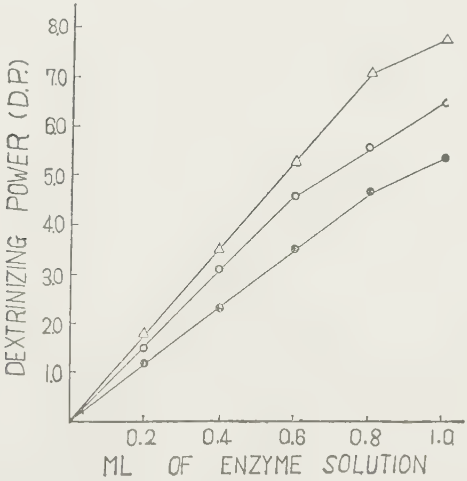

FIG. 2. Relation of enzyme concentration to dextrinizing power (D.P.).

●—● Purified malt amylase [α-type]; ○—○ crystalline Taka amylase [α-type]; △—△ crystalline sweet potato amylase [β-type].

D.P. was measured by the proposed method (see text).

Relation of Amylose Type to Dextrinizing Power (D.P.)—D.P. values for several amylase solutions at an arbitrary concentration obtained by the proposed method using P.E.-amyloses prepared from potato, corn and sweet potato starches are shown in Table I. It is obvious from the data shown that P.D. value of a given amylase solution is unaffected by the sources from which P.E.-amyloses were obtained. From similar experiments with potato P.E.- and B-amyloses it is clear that D.P. value is also unaffected by the methods of amylose preparation.

Table I

*Relation of P.E.-amyloses Prepared from Potato, Corn and Sweet Potato Starches to Dextrinizing Power (D.P.)**

Amyloses	Amylases				
	Crystalline bacterial amylase [α-type]	Crystalline Taka amylase [α-type]	Purified malt amylase [α-type]	Extract of powder of germinated rice grain	Crystalline seweet potato amylase [β-type]
Potato	3.0	4.9	2.6	3.3	0.9
Corn	3.0	5.1	2.6	3.5	0.9
Sweet potato	2.9	5.0	2.5	3.4	0.8

* Figure shows value of D.P. The concentration of each amylose is arbitrary.

Effect of Some Salts on the Color Intensity of Amylose-iodine Complex—As shown in Table II, the color of amylose-iodine complex measured under the proposed conditions is found to be unaffected by the presence of acetate buffer (pH 5.0), sodium chloride, calcium acetate, and ammonium sulfate at final concentration of $M/500$, $M/50$, $3\,M/1000$, and $4M/150$, respectively. Acetate buffer (pH 6.0) and phosphate buffers (pH 6.0 and pH 7.0) showed also no effect on the color intensity at the same concentration as that used in the case of acetate buffer (pH 5.0). Sodium chloride is well known as an activator of animal α-type amylases. However, the final concentration of this salt used as the activator in the reaction mixture is usually much lower than the concentration tested (1 M). Calcium salts have been frequently used in the course of amylase purification for the purpose of stabilizing amylase and of precipitating other proteins. Hobson *et al.* (*9*) showed that the presence of calcium ions (calcium chloride or calcium nitrate) at the concentration of 0.07 M in their reaction mixture, namely 0.0007 M in the dilute solution for the colorimetric reading, caused a slightly higher "Absorption Values" of amylose-iodine complexes. However, the author could not find such effect of calcium salts (calcium acetate, calcium chloride, and calcium nitrate) at the concentration of 0.003 M in the final dilute solution for colorimetric measurement. The concentration (0.15 M in the reaction mixture) corresponds to that which would be contained in the reaction

TABLE II

Effect of Some Salts on the Color Intensity of Amylose-iodine Complexes*

Amylose	Salt				
	—	Acetate buffer, pH 5.0	NaCl**	$Ca(CH_3COO)_2$**	$(NH_4)_2SO_4$**
	—	*M*/10***	*M****	15 *M*/100***	4 *M*/3***
	—	*M*/500****	*M*/50****	3 *M*/1000****	4 *M*/150****
4 *mg.*	0.370	0.368	0.366	0.362	0.370
2	0.185	0.184	0.182	0.181	0.185

* Colorimetric readings on logarithmic scale (optical densities).

** Each one contains *M*/500 acetate buffer (pH 5.0) in the dilute solution for colorimetric readings.

*** Concentration in the reaction mixture for D.P. determination (5 ml. total volume).

**** Concentration in the dilute solution for the colorimetric measurement (250 ml. total volume).

mixture when 1 ml. of 0.5 *M* calcium salt solution is used as the enzyme solution in the proposed method for D.P. determination. Ammonium sulfate has also been frequently used in the course of amylase purification as the salting out reagent of proteins. The concentration of this salt indicated in Table II corresponds to that would be contained in the reaction mixture when 1 ml. of aqueous solution saturated with ammonium sulfate (at 20°) is used as the enzyme solution in the proposed method.

Relation of the Glucose Concentration to the Color Intensity by Somogyi-Nelson's Method—Methods for the colorimetric determination of glucose with the aid of Somogyi's copper reagent (*11*) and Nelson's arsenomolybdate reagent (*12*) were tested at various concentration of glucose. The data given in Fig. 3 illustrate the fact that the color densities are in excellent agreement with Beer's law in the range between 0.1 and 0.5 mg. concentration of glucose. Reducing power of maltose measured by the method was found to be exactly equivalent to half of that of glucose.

Effect of Time on the Color Intensity by Somogyi-Nelson's Method—Nelson (*12*) and Somogyi (*11b*) reported that the arsenomolybdate reagent possesses the virtue of developing maximum color density almost instantaneously, care being taken to dissolve the cuprous oxide completely. Since it takes some time to dissolve completely the cuprous oxide formed after making the solution acid by the addition of arsenomolybdate reagent and after shaking the mixture, it would be desirable to allow the mixture to stand for about 20 minutes after shaking. However, it was found that the color faded out slowly when the solution was allowed to stand for more than 60 minutes

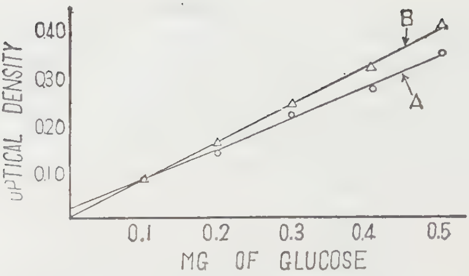

FIG. 3. Relation of the glucose concentration to the color intensity by Somogyi-Nelson's method.

●—● Somogyi's copper-phosphate-tartrate reagent (reagent A): ○—○ Somogyi's new copper-carbonate-tartrate reagent for colorimetry (reagent B).

Three ml. of reagent A or B[8], 0.5 ml. of 0.5 M acetate buffer of pH 5.0 and appropriate amount of glucose solution were mixed, the mixture was brought to a volume of 6 ml. and heated for 30 minutes in a boiling water bath. To the cooled solution were added 3 ml. of Nelson's arsenomolybdate reagent[9]. The solution was swirled and after 20 minutes made up to 100 ml. with water: Colorimetric readings were taken with a photoelectric colorimeter[6] fitted with a S_{50} filter[10] and a 20 mm.-glass cell. The measuring instrument was set to zero against a reagent blank which had been prepared by the same procedure.

before the dilution being made.

The blue color is stable for a unmber of hours after dilution being made. In the case where Somogyi's new copper-carbonate-tartrate reagent for colorimetry is used, relative color intensities remain unchanged even after standing overnight. However, it is desirable in the case where the copper-phosphate-tartrate reagent is used, to complete the colorimetric measurement within 6 hours after the dilution, because irregular color fading was observed in some case after standing overnight.

Effect of Amylose on the Colorimetric Deterimination of Glucose by Somogyi-Nelson's Method—In the case of amylase activity determination by the saccharimetric method using amylose as the substrate, unreacted amyloses are apt to remain in the solution and affect determination of reducing powers. Therefore, the effect of amylose on the chlorimetric determination of glucose by Somogyi-Nelson's method was checked. Three mg. each of amylose was added to an appropriate amount, 0.125–0.5 mg., of glucose in the reaction mixture. After treating the mixture with the copper-phosphate-tartrate reagent and the arsenomolybdate reagent in the usual means, color intensiries at various concentrations of glucose were compared with those of controls which had been prepared according to the same procedure, except that no addition of amylose was made. The data are shown in Fig. 4. It is obvious that this

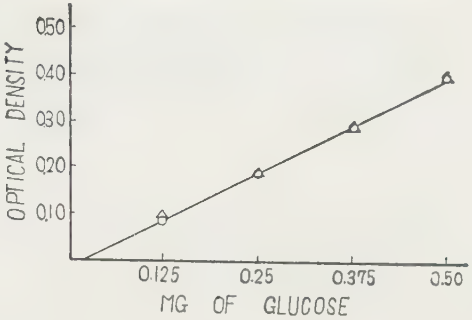

Fig. 4. Effect of amylose on the colorimetric determination of glucose by Somogyi-Nelson's method.

○—○ Glucose without amylose: △—△ glucose with 3mg. of amylose.

For the detailed procedure, see the foot-note of Fig. 3 and text. Copper reagent A is used.

amount of amylose causes slightly higher optical density at the lower concentration of glucose because of some clouding due to insolubility of amylose in acidic solution. However, this effect of amylose is not serious and reducing power of amylose itself is affected negligibly under these conditions. The concentration of amylose used in the experiment as shown in Fig 4. (3 mg. of amylose per 100 ml. of the final dilute solution for the colorimetric measurement) is the maximum concentration supposed to be present considering all amyloses remain unchanged, at which its concentration would be lower than these. Actually, however, it will be possible, therefore, to use this colorimetric method for the determination of S.P.

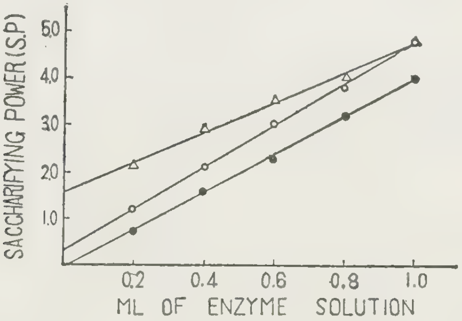

Fig. 5. Relation of enzyme concentration to saccharifying power (S.P.).

●—● Purified malt amylase [α-type]: ○—○ crystalline Taka amylase [α-type]: △—△ crystalline sweet potato amylase [β-type].

S.P. was measured by the proposed method (see text).

Relation of Enzyme Concentration to Saccharifying Power (S.P.)—The data given in Fig. 5 illustrate the fact that S.P. values measured by the proposed method are strictly proportional to the amount of enzyme present in purified malt amylase [α-type] and crystalline Taka amylase [α-type]. In the case of crystalline sweet potato amylase [β-type] it is very difficult to measure optical densities for S.P. determination at lower enzyme concentrations because of clouding of solutions. Accordingly S.P. values obtained at various concentrations of this amylase were found to be higher rather than those expected and had shown poor proportionality relation to the enzyme concentration. As shown in Fig 4. the blue color produced in the Somogyi-Nelson's colorimetric glucose determination was found to be unaffected by the presence of amylose even at the maximum—concentration, 3 mg. of amylose per 100 ml. of the final volume and it is rather difficult to explain the phenomenon. One of the probable causes may be that shorter chain amyloses produced by the action of enzyme are precipitated readily than the substrate amylose under these conditions and the other that a complex of amylose-amylase [β-type] protein is easily precipitated under these conditions.

Relation of Amylose Type to Saccharifying Power (S.P.)—S.P. values for several amylase solutions at an arbitrary concentration obtained by the proposed method using P.E.-amyloses prepared from potato, corn, and sweet potato starches are shown in Table III. It is obvious from the data shown that S.P. value of a given amylase solution is unaffected by P.E.-amyloses obtained from different sources. From similar experiments with potato P.E.-and B-amyloses it is clear that S.P. value is also unaffected by amyloses prepared by different methods.

TABLE III

*Relation of P.E.-amyloses Prepared from Potato, Corn, and Sweet Potato Starches to Saccharifying Power (S.P.)**

Amyloses	Amylases				
	Crystalline bacterial amylase [α-type]	Crystalline Taka amylase [α-type]	Purified malt amylase [α-type]	Extract of powder of germinated rice grain	Crystalline sweet potato amylase [β-type]
Potato	2.6	4.6	0.8	4.3	3.6
Corn	2.6	4.6	0.8	4.3	3.6
Sweet potato	2.5	4.6	0.8	4.3	3.6

* Figure shows value of S.P. The concentration of each amylase is arbitrary.

Effect of Some Salts on the Colorimetric Determination of Glucose by Somogyi-Nelson's Method—As shown in Table IV, the blue color of Somogyi-Nelson's colorimetric glucose determination was found to be unaffected by the presence of acetate buffer (pH 5.0), sodium chloride, calcium acetate and ammonium sulfate at concentration of $M/400$, $3M/100$, $M/2000$, and $M/5000$, respectively. Acetate buffer (pH 6.0) and phosphate buffers (pH 6.0 and pH 7.0) showed also no effect on the color intensity at the same concentration as that used in the case of acetate buffer (pH 5.0). However, the cuprous oxide was not formed after 30 minutes heating

TABLE IV

Effect of Some Salts on the Colorimetric Determination of Glucose by Somogyi-Nelson's Method$^{1)}$

Glucose		Salt							
		(a)	(b)	(c)	(d)		(e)		
		—	Acetate buffer pH 5.0	NaCl	$Ca(CH_3COO)_2$		$(NH_4)_2SO_4$		
		—	$5M/60^{2)}$	$M^{2)}$	$M/60^{2)}$	$M/600^{2)}$	$4M/3^{2)}$	$4M/300^{2)}$	$2M/300^{2)}$
mg.		—	$M/400^{3)}$	$3M/100^{3)}$	$M/200^{3)}$	$M/2000$	$M/25^{3)}$	$M/2500$	$M/5000^{3)}$
0.4	I$^{4)}$	0.28	0.28	0.28	—	0.28	—	—	0.28
	II$^{5)}$	0.28	0.30	0.30	卌$^{7)}$	—	卌$^{7)}$	—	—
		0.28	0.30	0.29	—	0.31	—	0.26	—
	III$^{6)}$	0.28	0.30	0.29	—	0.30	—	—	0.29
0.2	I$^{4)}$	0.14	0.14	0.13	—	0.14	—	—	0.13
	II$^{5)}$	0.14	0.14	0.13	卌$^{7)}$	—	卌$^{7)}$	—	—
		0.14	0.15	0.13	—	0.16	—	0.12	—
	III$^{6)}$	0.14	0.15	0.14	—	0.15	—	—	0.14

1) Somogyi's copper-phosphate-tartrate reagent is used for all experiments. Values of the color intensity are reading obtained on logarithmic scale (optical density).

2) Concentration in the reaction mixture for S.P. determination (total voume of 3 ml.).

3) Concentration in the dilute solution for the colorimetric measurement (100 ml. total volume).

4) Each one except (a) and (b) contains supplementary $5M/60$ acetate buffer of pH 5.0 before heating. The instrument is set to zero against a reagent blank without acetate buffer.

5) The measuring instrument is set zero against a reagent blank without acetate buffer.

6) Each one except (b) contains supplementary $5M/60$ acetate buffer of pH 5.0 before heating. The measuring instrument is set to zero against a reagent blank with acetate buffer.

7) Cuprous oxide is not formed after 30 minutes heating.

under the standard conditions in the presence of $M/6$ calcium acetate solution or $4M/3$ ammonium sulfate solution in 3 ml. of the reaction mixture and the color intensity after dilution to 100 ml. in the presence of $4M/300$ ammonium sulfate solution in 3 ml. of the reaction mixture was somewhat lower than that of the control in which ammonium sulfate is absent. These findings show that if each ml. of enzyme solutions is added to the reaction mixture, total 3 ml. volume, the enzyme solution which contains a solution of $M/2$ calcium acetate must be diluted 100 times and enzyme solution saturated with ammonium sulfate must be dliuted 200 times for the S.P. determination.

Values of D.P. and S.P. Per Microgram of Protein Nitrogen of Crystalline Taka Amylase [α-Type]—Values of D.P. and S.P. were measured by the proposed methods at various concentrations of Taka amylase [α-type] crystallized twice and the specific activities (amylase units per microgram of protein nitrogen) were calculated. Values obtained graphically were found to be 480 D.P./γ pN 110 S.P./γ pN, respectively (see Figs. 6 and 7). The specific D.P. of crystalline sweet potato amylase [β-type] obtaimed by the similar procedure was 12 D.P./γ pN (Fig 8). The specific S.P. of this enzyme was not determined because of inaccurate values obtained due to clouding of solution during colorimetric measurement as mentioned above.

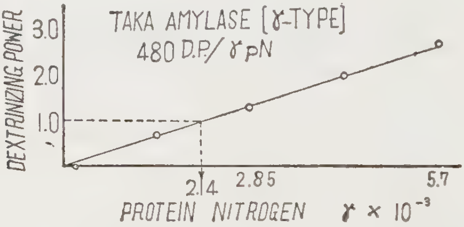

FIG. 6. Determination of D.P. value per microgram of protein nitrogen of crystalline Taka amylase [α-type].

Ten thousand times diluted stock solution of twice crystallized Taka amylase [α-type] (0.286 γ of protein nitrogen per ml.) was diluted 50/1, 100/1.5, 100/1, and 200/1 times, respectively. Each ml. of the diluted solutions was used as the enzyme solution for D.P. determination by the proposed method (see text). The curve was made with an average of 3 values obtained from separate duplicate determinations.

Ten thousand times diluted stock solution of twice crystallized Taka amylase [α-type] (0.286 γ of protein nitrogen per ml.) was diluted 25/1, 100/3.5, 100/3, 100/2.5, 50/1, 100/1.5, 100/1, and 200/1 times, respectively. Each ml. of the diluted solutions was used as the enzyme solution for S.P. determination by the proposed method (see text). Somogyi's copper-phosphate-tartrate reagent was used. The curve was made with an average of 5 values from separate duplicate determinations.

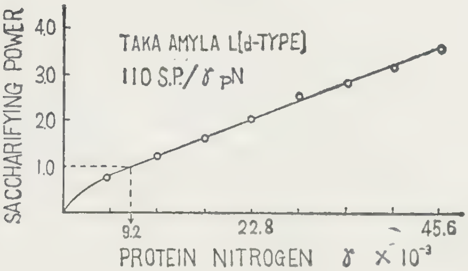

Fig. 7. Determination of S.P. value per microgram of protein nitrogen of crystalline Taka amylase [α-type].

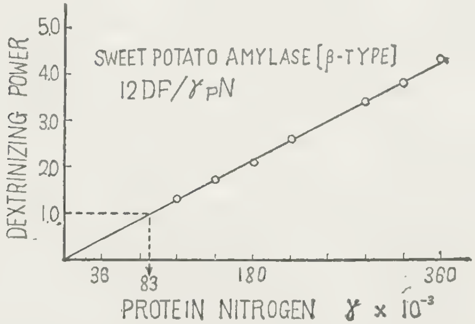

Fig. 8. Determination of D.P. value per microgram of protein nitrogen of crystalline sweet potato amylase [β-type].

Each ml. of 25/2.5, 25/2, 25/1.5, 50/2.5, 25/1, and 50/1.5 times diluted solutions of 100 times diluted stock solution of twice crystallized sweet potato amylase [β-type] (3.6 γ of protein nitrogen per ml.) and 1.5 ml. of 25/1.5 times diluted solution of the same 100 times diluted solution were used as the enzyme solution for D.P. determination by the proposed method (see text). The curve was made with an average of 3 values obtained from separate duplicate determination.

DISCUSSION

From the data given above, it is clear that the proposed methods for D.P. and S.P. determinations allow relatively rapid and easy measurement of amylase activites with a considerable degree of accuracy with small amounts of enzymes and reagents, although there remain several points for further consideration. Advantages and limitations of these methods are as follows:

D.P. Determination Method—The S_{70} gelatin filter[7], transmitting light of wave length 700 mμ. provided with the AKA photoelectric colorimeter (Kotaki-Seisakusho, Tokyo) was employed in the measurement of light absorbance of amylose-iodine complexes in the D.P. determination. The chief reasons for the employment of this weve length for the colorimetric measurement are mentioned below. (a) Because 0.002 per cent I_2–0.02 per cent KI solution scarcely shows light absorption under these conditions, the instrument being set against water instead of the I_2–KI solution. It is convenient for daily analysis. (b) Because shorter chain amyloses which are produced by the action of amylase and which exhibit light absorption spectra with a maximum absorption at shorter wave lengths show less absorption under these conditions. Consequently, it might be expected that good proportionality between enzyme activity and enzyme concentration would be held over a wide range of enzyme concentrations. While amylose-iodine complexes have the maximum absorption at about 600–650 mμ. (*23*), McCready *et al.* (*10*) measured blue values of amyloses with K–66 filter furnished with the Klett-Summerson photoelectric colorimeter, because 85 per cent of the light transmitted through this filter had a wave length of 640–700 mμ, which was close to Simerl and Browing's (*24*) optimum wave length for the estimation of this blue color. In this case the instrument was set to zero against the I_2-KI blank which had a light yellow color due to the iodine. Hobson *et al.* (*9*) used Ilford gelatin filter 603 transmitting light of wave length 680 mμ. furnished with a Spekker photoelectric absorptiometer (Hilger and Watts, London) to measure the changes of blue values of amyloses for α-amylase activity measurement in their study on amylases of rumen bacteria, because of the similar reasons mentioned by the author. Kung *et al.* (*6*) measured the change of blue values of amyloses for the measurement of activities of several α-type amylases with a Beckman spectrophotometer and with a Cary recording spectrophotometer at the wave lengths of maximum absorption and slso at 610 mμ. At any rate it remains to be studied to find a proper wave length to be used for measurement of blue values of amyloses in the determination of dextrinizing power. The intensity of absorption of amylose-iodine complexes decreases without any shift of the absorption peak or change of the shape of the curve of absorption spectra as the hydrolysis proceeds during the action of β-type amylase (*23b*, *25*). In the case of α-type amylase the peaks of the absorption spectra shift toward shorter wave lengths (*6*, *23b*). It will then be expected that D.P. values of α-type amylases are affected more strongly than those of β-type amylases by wave length employed and properties of the filter used. Even though we can determine the optimun wave length as well as the other optimum conditions for the determination of dextrinizing power of a given amylase, differences in D.P. values may result from variations introduced by the properties of a filter used unless the spectrophotometer is used. This is the limitation in D.P. determination to be considered in obtaining a universal unit of amylase activity.

Differences in D.P. value of a given amylase solution measured by the use of various types of amylose as the substrate are somewhat greater than those found in S.P. values obtained in the similar experiments (see Tables I and III). However, a maximum difference observed was less than 5 per cent when D.P. was measured under the optimum conditions for this method (*i.e.*, in the range of D.P. 3–4). Moreover, D.P. value is unaffected by the presence of various salts frequently contained in amylase solutions at fairly high concentrations as shown in Table II. These are good features of the proposed D.P. determination method.

In the consideration of the concentration of the substrate amylose, it seems to be better to use lower concentrations for D.P. determination of α-type amylase, instead of 0.1 per cent used in the present experiment. In this case amylase reaction is limited to the rapid phase which is terminated at about the achroic stage and the subsequent fission of the achroic products scarcely occurs in very dilute amylose solution as described by Hopkins *et al.* (*7a*). Moreover, the lower concentration of amylose will eliminate the possibility of retrogradation more completely, even though the tendency of retrogradation which necessarily affects on D.P. value can not be recognized under the proposed conditions. On the othet hand, however, low concentration of the substrate means low enzyme concentration under a given condition, and this is not desirable to provide lower substrate concentration for the measurement of activity of amylase which would be inactivated by dilution. In such a case it will be better to use a short reaction time and a high amylase conentration as reaction conditions in order to prevent the inactivation of the amylase by dilution.

S.P. Determination Method—Dickson *et al.* (*26*) showed that diastatic powers measured by the ferricyanide method were not affected by the use of various types of soluble starch. However, it is known that activity of serum amylase estimated by Somogyi's saccharimetry (*27*) were affected by various types of starch (*28*). Andersch (*8*) succeeded in developing a colorimetric method to use β-amylose eluted from potato starch and adsorbed on cotton as the substrate by modifying the method of the Somogyi's serum amylase determination. The proposed method for S.P. determination is a micromethod applicable for various types of amylase by using well defined amylose as the substrate. It is an excellent feature of this method that S.P. value for a given amylase solution is never affected by amyloses obtained from different sources or prepared by different procedures. Although clouding did not interfere with the colorimetric measurement under the proposed conditions in the case of α-type amylases, it was found difficult to take measurement of optical densities of crystalline sweet potato amylases [β-type] at the lower concentrations because of clouding of the solutions. Consequently, S.P. values obtained at various concentrations of this amylase were found rather high and showed poor proportionality to the enzyme concentration. This difficulty will be eliminated, however, by making the concentration of amylose lower than that used in the proposed method.

It is desirable in S.P. determination to have the reaction glucose, maltose, amylooligosaccharides or amylose dextrins, and natural amyloses to be strictly stoichiometric when their reducing values are measured by a given method. A few of such methods for the determination of reducing values had been suggested for the estimation of molecular weights of polysaccharides (*29*), however, they were found to be inadequate for this purpose by some workers (*30*). Modified methods of Sumner's (*31*) and of Somogyi-Nelson's (*11*, *12*) methods have been widely adapted to the colorimetric determination of reducing substances formed during amylase action. Recently, Smith *et al.* (*32*) reported a method for determination of activities of serum and urine amylases by the saccharimetric method, using Sumner's 3,5-dinitrosalicylic acid reagent. Teller (*33*) reported a method for the determination of serum amylase using Somogyi-Nelson's reagent. Both methods are fairly adequate as the micromethod using soluble starch as the substrate. In the author's method, Somogyi's and Nelson's reagents are used for determining reducing substances in the presence of amylose as the substrate. The method by Somogyi's new copper-carbonate-tartrate reagent (*11b*) is superior to the copper-phosphate-tartrate reagent (*11a*) owing to the fact that the former reagent is stable for storage, gives reproducible results being in excellent agreement with Beer's law, and developes stable color.

In the determination of amylase activity by the proposed S.P. determination method, amounts of reducing substances formed in the course of amylase action are calculated as glucose. It is known that maltose is a sole fission product by the acion of β-type amylase on amylose (*7b*, *c*) and glucose is a sole fission product by amyloglucosidase type amylase (*34*). In these cases it will then be reasonable to calculate reducing values of fission product as maltose and glucose, respectively, in order to obain S.P. values for each amylase. On the other hand, various fission products (dextrins, oligosaccharides, maltose, and glucose) are produced by the action of α-ype amylase on the same substrate. Since an accurate determination of each fission product is very difficult and the reactions involved between these fission products and the reagent used are not stoichiometric as mentioned above, it will be reasonable to calculate the reducing values as glucose in these cases.

Thus the reducing values are calculated as glucose since the standard curves were prepared by taking known concentrations of glucose. Accordingly S.P. value obtained for a given amylase solution never be affected by variations pertinent to the measuring instrument used such as wave length or a type of filter used. However, as shown in Table IV S.P. value is affected by the presence of some salts which are frequently contained in amylase solutions. It is impossible to eliminate the effect completely unless more suitable reagent for the determination of reducing substances will be found, because the effect depends on the properties of Somogyi's copper reagent.

At any rate, if we take precautions in modifying the limitations observed for respective amylase, the proposed method can be adapted to D.P. and S.P. determinations of various types of amylase.

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SUMMARY

Procedures have been presented for the colorimetric microdetermination of dextrinizing power (D.P.) and saccharifying power (S.P.) of amylases by the use of amylose as the substrate.

For D.P. determination, decrease in amount of the substrate was measured by the change of blue values of amylose-iodine complexes.

For S.P. determination, reducing substances formed were determined by using a combination of Somogyi's copper reagent and Nelson's arsenomolybdate reagent.

Studies were undertaken to examine effects on D.P. and S.P. values of a given amylase solution due to difference in sources and different methods of preparation of amylose and also the presence of some salts.

Advantages and limitations of the proposed procedures are discussed in detail.

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COMPARATIVE STUDIES ON THE SUSCEPTIBILITY OF PHOTOSYNTHESIS, THE HILL REACTION AND THE CATALASE REACTION TOWARDS VARIOUS INHIBITORS

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Among the thousands of biochemical and physiological reactions shown by green plants, there are three principal processes that are accompanied by the evolution of oxygen; namely, photosynthesis, the Hill reaction and the catalatic decomposition of hydrogen peroxide. It has now become a general consensus that the evolution of oxygen in the Hill reaction is brought about by the same mechanism as the oxygen evolution in normal photosynthesis, whereas opinions differ as to whether the catalatic activity of cells has any bearing on the two other processes.

One of the effective methods for the elucidation of the relationship existing between these processes is the inhibition technique, in which comparison is made of the susceptibility of each process towards various specific substances. Important in this connection is the fact reported by Tamiya *et al.* (*1*, *2*, *3*, *4*) that the inhibition of photosynthesis caused by various poisonous substances shows characteristic quantitative relationships according to the nature of the inhibitor applied. If the degree of inhibition (H) is defined by $H=1-(V_g/V)$ (where V_g and V are the velocities of photosynthesis in the presence and absence, respectively, of the inhibitor), H was found to be the functions of concentration of the inhibitor ([G]) in three different manners as follows:

(1) In the first type, which was found in the case of cyanide and phenyl urethane, the relation between H and [G] follows the equation

$$H=\frac{[G]}{\phi+[G]} \qquad (1)$$

where ϕ is a constant corresponding to the concentration of inhibitor causing 50 per cent inhibition. This mode of inhibition may be termed as the inhibition of the "first order";

(2) In the second type, which may be called the inhibition of the

"second order", the relation observed was:

$$H=\frac{[G]^2}{\phi^2+[G]^2} \qquad (2)$$

This type of inhibition was found in hydroxylamine, azide, oxygen and ethyl urethane;

(3) The third type, which may be regarded as an intermediary type between the other two, is characterized by the relation.

$$H=\frac{\phi_2[G]^2+\phi_1[G]}{\phi_2[G]^2+\phi_1[G]+\phi_0} \qquad (3)$$

where ϕ_0, ϕ_1 and ϕ_2 are constants. To date, this type of inhibition has been observed only in certain styryl and cyanine dyes.

Apart from the interpretation of these three types of inhibition—which was attempted by Tamiya in his theory on the mechanism of photosynthesis (*1*)—it seems worth while to investigate quantitatively in what manner the other oxygen-producing processes, the Hill reaction and the catalatic H_2O_2-decomposition, will be affected by the substances known to be inhibitory upon photosynthesis.

METHODS

Materials—Chlorella ellipsoidea was cultured as described in previous reports from our laboratory (*5*). The cells were centrifuged out of the culture medium, washed three times with $M/500$ K_2SO_4. and then suspended in the same solution. Some comparative studies were made with chloroplast fragments ("grana") which were isolated from leaves of *Spinacia oleracea** by the following procedure. About 50 g. of fresh leaves were dipped in distilled water at 2–4°, and after being illuminated for 1–2 hours with a 200 watt incandescent lamp at a distance of 30 cm., they were macerated for one minute in an ice-cooled blendor with about 100 ml. of phosphate buffer ($M/30$, pH 7.5). Cell fragments were removed by centrifugation for 5 minutes at *ca.* $170\times g$, followed by filtration through a plug of glass wool in a funnel. The green suspension obtained was centrifuged for 5 minutes at *ca.* $3{,}400\times g$, and the sedimented chloroplasts were resuspended in $M/30$ phosphate buffer (pH 7.5) containing 50 per cent glycerol. The suspension contained about 0.2–0.5 mg. chlorophyll per ml.

Measurement—Photosynthesis and the Hill reaction were measured manometrically with Barcroft differential manometer at 15°, pH 7.0. The light intensity at the level of reaction vessels was about 10,000 lux or higher. Gas space was usually filled with pure nitrogen which had been freed from oxygen and carbon dioxide. With intact *Chlorella* cells the manometric measurement of the Hill reaction required special precaution for excluding the simultaneous occurrence oxygen evolution by photosynthesis. For this purpose buffers and other solutions were previously freed from carbon dioxide as much as possible, and the trace of carbon dioxide left was removed by letting the algae photosynthesize under illumination for 40–60 minutes before the addition of oxidant*. The Hill reagent used for intact algal cells was *p*-benzoquinone which had been purified by sublimation. The end concentration of the quinone added was $10^{-2.5}$ M which has been confirmed to be optimal and " saturating " for the reaction. At this concentration of quinone, oxygen evolution occurred at a steady rate until it ceased at the point of exhaustion of the oxidant corresponding to the relation: $2\ \text{Quinone} + 2H_2O \rightarrow 2\ \text{Hydroquinone} + O_2$. The steady rate was found to be proportional to the quantity of algal cells up to 30 mg. of cells per vessel.

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*In most experiments, we used spinach procured directly from the field. Spinach obtained from the market showed a considerable fluctuation in activity.

For measuring the catalatic decomposition of H_2O_2, H_2O_2 solution was added to algal suspensions in phosphate buffer of pH 7.0, and the reaction was followed by titrating H_2O_2 with $M/15$ $KMnO_4$. The final concentrations of H_2O_2 and phosphate were 0.015 and 0.05 M, respectively.

Chlorophyll content in algal cells was determined by measuring spectrophotometrically the quantity of pheophytin formed when the algal suspension was treated with 10 per cent HCl (See (*7*, *8*)).

EXPERIMENTAL RESULTS

Before presenting the data of inhibition experiments, it seems pertinent to compare the rate of O_2-evolution by the three processes in question in the absence of inhibitors. While the *Chlorella* cells grown under our culture conditions showed a fairly constant rate of photosynthesis,** their activity for the Hill reaction varied, for some reason unknown at present, in a rather wide range. A still wider fluctuation in the activity for the Hill reaction was observed in isolated grana, which, according to the author's experience for several years, show high activity in winter and very low—sometimes even no—activity in summer. In Table I, the relative rates of O_2-evolution in these processes are given in terms of mole O_2 per second per 1 g. (dry weight) of each sample. For comparison, the rate of the Hill reaction shown by isolated grana is also given in terms of $Q_{O_2}^{\text{Chl}}$-value, *i.e.*, the volume (in cu mm.) of O_2 liberated per hour per mg. of chlorophyll contained in the sample. It should be remarked that, in the case of intact algal cells, the maximum rate of O_2-evolution by the Hill reaction is only about

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*We could not corroborate the statement by Clendening and Ehrmantraut (*6*) that the photosynthetic activity of intact cells is totally suppressed in the presence of the Hill reagent such as quinone or ferric salts. According to our observations, the suppressing effect of the Hill reagent upon photosynthesis is only partial, while the Hill reaction in the presence of quinone proceeds at practically the same rate whether photosynthesis is simultaneously going or not, according to the presence or absence of CO_2.

** See, for example, Tamiya and Chiba (*5*).

a half of the corresponding photosynthetic rate. The figures given for the catalatic H_2O_2-decomposition should be construed in a different way than those given for other processes, because its rate, measured in ordinary experimental conditions, depends primarily on the concentration of H_2O_2 present at each moment.

TABLE I

Rates of Various O_2-liberating Reactions in the Absence of Inhibitors

Material	Reaction	Condition	O_2-evolution in mole per second per 1 g. $\times 10^1$
Chlorella	Photosynthesis	CO_2-and light-saturation, at 15° and pH 7.0	11.6±0.5
	Catalatic H_2O_2-decomposition	In the presence of 0.015 *M* H_2O_2, at 0° and pH 7.0	5.5
	Hill reaction	Quinone as oxidant, light-saturation, at 15° and pH 7.0	In most cases 1–2 Maximum 5.6 (Corresponding to $Q_{O_2}^{Chl}=1{,}230$)
Spinach grana	Hill reaction	Quinone as oxidant, light-saturation, at 15° and pH 7.0	$0\sim 0.9$ (Corresponding to $Q_{O_2}^{Chl}=0\sim 700$)
		Hill's ferric salts mixture as oxidant, light-saturation, at 15° and pH 7.0	$0\sim 0.9$ (Corresponding to $Q_{O_2}^{Chl}=0\sim 700$)

Hydroxylamine—It has been shown previously that the inhibition of algal photosynthesis caused by hydroxylamine is of the second order, while the inhibition of catalatic H_2O_2-decomposition by the same organism is of the first order. Experiments using intact *Chlorella* cells as well as isolated grana showed that the hydroxylamine inhibition of the Hill reaction is also of the second order.

A characteristic feature of hydroxylamine action on both photosynthesis and the Hill reaction by intact algal cells is that the inhibition occurs with an induction period lasting more than 30 minutes (at 15°), after which the degree of inhibition attains a definite value. Whereas the value ϕ for the Hill reaction by intact algal cells did not change appreciably with the activity of each algal sample, the corresponding value shown by isolated grana varied considerably, becoming increasingly larger with the decrease of the activity of the sample. The results summarized in Table II show to what extent the inhibition of the Hill reaction caused by $10^{-3.0}$ *M* hydroxylamine varied with the activity of the grana samples used. The lack of agreement among several authors (*9*, *10*, *11*, *12*, *14*) concerning the effect of hydroxylamine upon the Hill reaction shown by isolated chloroplast is obviously derived from data based upon the use of samples of different activity.

TABLE II

Inhibitory Effect of Hydroxylamine upon the Hill Reaction of Isolated Grana as It Changes with the Activity of the Grana Samples Used

O_2 evolved in 20 min. in the absence of NH_2OH (*cu. mm.*)	Chlorophyll content (*mg./vessel*)	$Q_{O_2}^{Chl}$	O_2 evolved in 20 min. in the presence of 10^{-3} *M* NH_2OH (*cu. mm*)	Degree of inhibition (H) (%)	Oxidant used
108	0.25	1300	0	100	Quinone
77	0.30	770	0	100	Hill's mixture
69	0.32	650	24	65	//
53	0.34	450	20	53	//
50	0.40	370	39	22	//
38	0.46	290	33	13	//
25	0.40	190	21	15	Quinone

In Fig. 1, the H-pG-curves of photosynthesis, the Hill reaction and the catalatic H_2O_2-decomposition by intact *Chlorella* cells as well as the Hill reaction by isolated grana are compared. The values of ϕ and the order of inhibition from these curves are listed in Table III. The values for isolated grana are those obtained with the most active sample.

The noteworthy facts brought out in this experiment are as follows:

(1) Hydroxylamine exerts a second order inhibition both upon photosynthesis and the Hill reaction, whereas it causes a first order inhibition in the case of the catalatic H_2O_2-decomposition;

(2) The ϕ-values for photosynthesis and the Hill reaction are practically the same in intact algal cells, being considerably larger than the corresponding value for the catalatic H_2O_2-decomposition by the same cells;

(3) The ϕ-value for the Hill reaction of isolated grana is independent of the oxidant used, although it changes considerably with the activity of grana to show the Hill reaction.

Azide—The mode of action of azide is similar to that of hydrxylamine in so far as their action upon photosynthesis and the catalatic H_2O_2-decomposition is of second and first order, respectively. A marked difference between these two inhibitors lies in the fact that the azide inhibition of the Hill reaction is of the first order, instead of

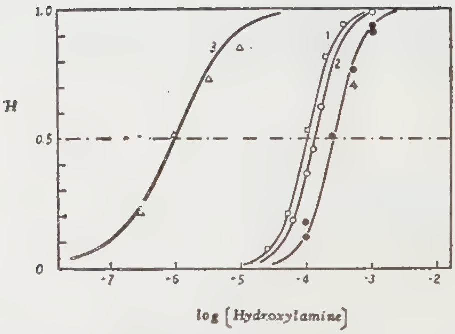


FIG. 1. Inhibition-pG-curves of hydroxylamine in photosynthesis, the Hill reaction and catalatic H_2O_2-decomposition.

Curve 1. Pohtosynthesis by intact *Chlorella* cells. (pH 7.0, 15°) (□——□). Curve 2. The Hill reaction by intact *Chlorella* cells. (pH 7.0, 15°, quinone) (○——○). Curve 3. H_2O_2-decomposition by intact *Chlorella* cells. (pH 7.0, 15°) (△——△). Curve 4. The Hill reaction by isolated grana (pH 7.0, 15°, quinone) (●——●).

TABLE III

The Values of and the Order of Inhibition in the Hydroxylamine Inhibition of Various O_2-liberating Reactions (at pH 7.0 and 15°)

Material	Reaction	Condition	$-\log_{10}\phi$	Order of inhibition
Chlorella	Photosynthesis	CO_2-and light-saturation	4.0	2
	Hill reaction	Light-saturation, quinone as oxidant	3.9	2
	Catalatic H_2O_2-decomposition	In the presence of 0.015 M H_2O_2	6.0	1
Spinach grana (most active sample)	Hill reaction	Light-saturation, quinone as oxidant	3.6	2
		Light-saturation, Hill's mixture as oxidant	3.6	2

being of the second order as it was in the case of hydroxylamine. The H-pG-curves obtained with azide are shown in Fig. 2 and the values of ϕ and the order of inhibition estimated from these curves are summarized in Table IV.

Cyanide—Unlike hydroxylamine and azide, cyanide exerts a first order inhibition upon photosynthesis. The same is true in the cyanide inhibition of catalatic H_2O_2-

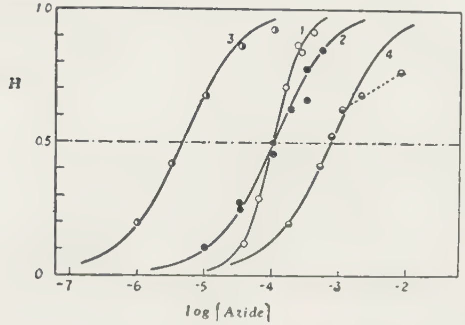


FIG. 2. Inhibition-pG-curves of azide in photosynthesis, the Hill reaction and catalatic H_2O_2-decomposition.

Curve 1. Photosynthesis by intact *Chlorella* cells (pH 7.0, 15°) (○——○). Curve 2. The Hill reaction by intact *Chlorella* cells (pH 7.0, 15°) (quinone) (●——●). Curve 3. H_2O_2-decomposition by intact *Chlorella* cells (pH 7.0, 15°) (◑——◑). Curve 4. The Hill reaction by isolated grana. (pH 7.0, 15°) (quinone) (◒——◒).

TABLE IV

The Value ϕ and the Order of Inhibition in Azide Inhibition of Various O_2-liberating Reactions (at pH 7.0 and 15°)

Material	Reaction	Condition	$-\log_{10}\phi$	Order of inhibition
Chlorella	Photosynthesis	CO_2-and light-saturation	4.0	2
	Hill reaction	light-saturation, quinone as oxidant	4.0	1
	Catalatic H_2O_2-decomposition	in the presence of 0.015 M H_2O_2 (4°)	5.4	1
Spinach grana	Hill reaction	light-saturation, quinone as oxidant	3.2	1

decomposition by intact algal cells (*1*, *2*) (See Fig. 3).

In investigating the effect of cyanide upon the Hill reaction, we must take into account the possibility that there might occur some sort of reaction between cyanide and the Hill reagent added, which may result in lowering the effective concentration of cyanide in the reaction mixture. To clarify this point, the effect of quinone ($10^{-2.5}$ M as it is used as the Hill reagent) upon the H-pG-relationship of cyanide against catalatic H_2O_2-decomposition by intact *Chlorella* cells was investigated. It was revealed that, as shown in Table V, the ϕ-value was slightly—by a factor of 3—increased by the addition of quinone, indicating that a certain fraction of cyanide added was rendered ineffective by the effect of quinone. This effect is, however, so insignificant that we may ignore it in studying the effect of cyanide upon the Hill reaction using quinone as the oxidant.

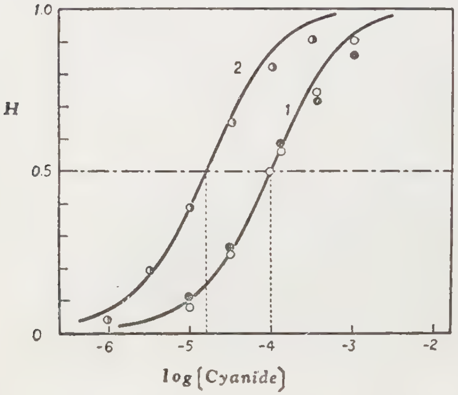


FIG. 3. Inhibition-pG-curves of cyanide in photosynthesis and the catalatic H_2O_2-decomposition by intact *Chlorella* cells.

Curve 1. Photosynthesis (pH 7.0 25°) (○——○) and the catalatic H_2O_2-decomposition (pH 7.0, 25°) (●——●). Curve 2. The catalatic H_2O_2-decomposition (pH 7.0, 4°) (◑——◑).

TABLE V

Effect of Quinone upon the Inhibition-pG-curves of Cyanide against Catalatic H_2O_2-Decomposition by Intact Chlorella Cells

	$-\log_{10} \phi$
Without of quinone	4.4
With quinone	4.05

* Concentration of quinone used: $10^{-2.5}$ *M*.
* pH 7.0, 15°

Experiments using intact *Chlorella* cells showed that the Hill reaction (with quinone as oxidant) was not affected at all by $10^{-3.0}$ *M* cyanide. Increase of cyanide concentration up to $10^{-2.0}$ *M* yielded occasionally some indication of inhibition, which was, however, so fluctuating that we could hardly get concrete quantitative data from those observations.

A peculiar phenomenon was observed when isolated grana were used as the material of the Hill reaction. When cyanide was added in the concentration of $10^{-2.5}$–$10^{-2.0}$ *M*, the gas evolution by the Hill reaction occurred almost normally in the first 5–15 minutes, after which, however, it ceased suddenly and was followed by a vigorous oxygen absorption with a velocity which was larger at higher cyanide concentrations (See Fig. 4).

The O_2-absorption did not occur in the dark and in nitrogen atmosphere, indicating clearly that it is due to a photochemical oxidation of some substance. Such a process caused by cyanide was not observed when intact *Chlorella* cells were used as material.

At any rate, it may be concluded that the Hill reaction is not affected at all by cyanide in such a concentration as it inhibits completely photosynthesis and the catalatic H_2O_2-decomposition.

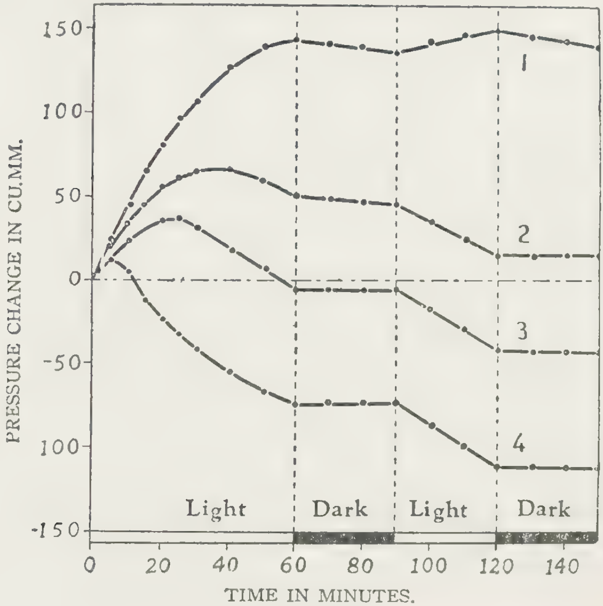


Fig. 4. Photo-oxidation caused by cyanide in the suspension of spinach grana.

Spinach chloroplast. Chlorophyll contents: 0.4 mg. per vessel. H-acceptor: Quinone. Gas-phase: Air.

Curve 1. Control. Curve 2. With $10^{-3.0}$ *M* cyanide. Curve 3. With $10^{-2.5}$ *M* cyanide. Curve 4. With $10^{-2.0}$ *M* cyanide.

2, 4-*Dinitrophenol*—2, 4-dinitrophenol has been found to suppress photosynthesis (*13*) as well as the Hill reaction (*14*). According to MacDowall (*14*) its effect on the Hill reaction becomes stronger when the light intensity was lowered.

Experiments with intact *Chlorella* cells showed that this substance exerts a second order inhibition both upon photosynthesis and the Hill reaction (see Fig. 5), while it does not affect the catalatic O_2-evolution even at the concentration as high as $10^{-2.5}M$. The ϕ-value for photosynthesis and the Hill reaction were found to be $10^{-3.6}$ *M*, and $10^{-3.2}$–$10^{-3.4}$ *M*, respectively. Contrary to the statement made by MacDowall, the inhibition of the Hill reaction caused by this substance was independent of the light intensity applied. For example, the following results were obtained with $10^{-3.0}M$

dinitrophenol:

Light intensity in lux:	1,400	6,000;
Degree of inhibition :	0.83	0.85.

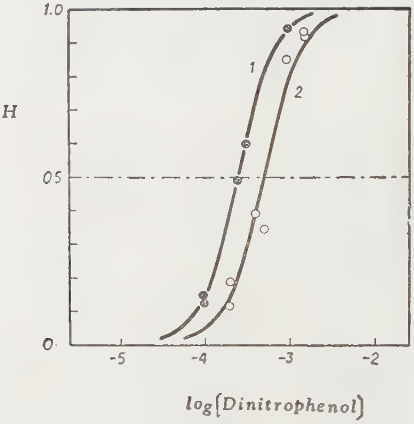


FIG. 5. Inhibition-pG-curves of 2,4-dinitrophenol in photosynthesis and the Hill reaction.

Curve 1. Photosynthesis by intact *Chlorella* cells (pH 7.0, 15°) (●——●). Curve 2. The Hill reaction by intact *Chlorella* cells (pH 7.0, 15°) (○——○).

DISCUSSION

The results obtained in this study are briefly summarized in Table VI.

TABLE VI

Comparison of the Mode of Action of Inhibitors upon Different O_2-evolving Processes Shown by Intact Chlorella Cells

(Figures given in parentheses indicate the "order" of inhibitory action).

	Photosynthesis	Hill reaction	Catalatic H_2O_2-decomposition
Hydroxylamine	+ (2)	+ (2)	+ (1)
Azide	+ (2)	+ (1)	+ (1)
Cyanide	+ (1)	−	+ (1)
2,4-Dinitrophenol	+ (2)	+ (2)	−

+: Inhibition, −: No inhibition.

In comparing the effect of an inhibitor upon an over-all process (A) with that on a simpler process (B), whose interposition in the mechanism of the former is investigated by the inhibition technique, we should take the following basic principles into account: (1) If a given inhibitor inhibits both processes A and B to almost the same degree, there is a strong possibility that process B represents a rate-determining intermediate step in process A; (2) If a given inhibitor inhibits process B to a considerably weaker degree than process A, the former is either (i) an intermediate step (besides another step which is sensitive towards the action of the inhibitor) or (ii) is not an intermediate step in process A; (3) If the inhibitor inhibits process B to a considerably stronger degree than process A, it is justifiable to conclude that process B is not involved in process A.

Based on these considerations we may draw the following conclusions from the data summarized in Table VI.

(1) In all probability, the evolution of oxygen by the Hill reaction is accomplished by the same mechanism as in ordinary photosynthesis. Noteworthy is the fact that hydroxylamine and 2,4-dinitrophenol attack the Hill reaction and photosynthesis with the same "order" —second order— and with approximately equal ϕ-values. (The fact that the Hill reaction is inhibited by 2,4-dinitrophenol seems to indicate the presence of some phosphorylation reaction in its mechanism.)

(2) The fact that cyanide inhibits photosynthesis but not the Hill reaction, indicates that photosynthesis involves some CN-sensitive reaction step other than the Hill reaction.

(3) Strong sensitivity of catalase towards cyanide indicates that it does not function in the Hill reaction, although the possibility of its participation in some other reaction step of photosynthesis is not entirely precluded. However, the situation seems to speak strongly against the assumption of catalase as an enzyme involved in photosynthesis, in so far as we assume that the enzyme functions in the reaction-phase of O_2-liberation. If catalase should function in some other step in the mechanism of photosynthesis, we have to assume that its mode of action is fundamentally different from that which we observe when it decomposes hydrogen peroxide.

The question now arises how it can be explained that azide exerts inhibition of a different "order" upon photosynthesis and the Hill reaction. Quite apart from this question it is rather strange that photosynthesis is affected in the manner of the second order by quite a number of substances such as hydroxylamine, azide, ethyl urethane, oxygen and

2,4-dinitrophenol. Survey of the literature has revealed that almost all inhibitory phenomena brought about in simple enzymatic reactions in vitro by the effect of reversible inhibitors are of the first order. The latter fact may be explained by a simple assumption ; that the inhibitor (G) combines reversibly with the enzyme (E) to form an inactive complex (EG) in the manner.

$$E+G \rightleftarrows EG.$$

In his theory of photosynthetic mechanism, Tamiya (*1*) assumed that the second order inhibition of photosynthesis is due to the bimolecular fixation by inhibitors of some enzyme involved in the Blackman reaction, namely

$$\begin{aligned} &E+G \rightleftarrows EG \\ &EG+G \rightleftarrows EGG. \end{aligned}$$

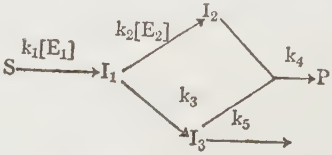
Steady state kinetics based on such an assumption leads to the following equation relating the degree of inhibition and the concentration of inhibitor.

$$H=\frac{\phi_2[G]^2+\phi_1[G]}{\phi_2[G]^2+\phi_1[G]+\phi_0}$$

This equation corresponds to the inhibition of the "intermediate type" mentioned in the introduction, but transforms into the equation for the second order inhibition, if ϕ_1 is sufficiently small compared with $\phi_2[G]$.

Apparently, however, the sites of attack by such diverse kinds of inhibitors as hydroxylamine, oxygen, ethyl urethane and 2, 4-dinitrophenol can hardly be thought to be the same. Moreover, the fact that the enzymes are only rarely inhibited in the manner of the second order makes the assumption of bimolecular fixation rather implausible as an explanation of the second order inhibition of photosynthesis.

It should be pointed out that, if a process involves a number of intermediate steps, there are possibilites that a second order inhibition of the over-all reaction is brought about as a result of the first order inhibition of some of the intermediate steps involved.* The following reaction mechanism represents one of the simplest examples of such cases.



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* This remark and the following example were suggested by Dr. H. Tamiya.

In this schema, S is the substrate, P the product, X a by-product, I_1, I_2 and I_3 are intermediate substances. As indicated on the arrows, the reaction rate of each step is k_1 $[E_1]$, k_2 $[E_2]$, k_3, k_4 and k_5, respectively, where $[E_1]$ and $[E_2]$ are concentrations of the enzymes which are affected by an inhibitor G in the manner of the first order as follows:

$$E_1 + G \overset{K_1}{\rightleftarrows} E_1G$$
$$E_2 + G \overset{K_2}{\rightleftarrows} E_2G$$

where K_1 and K_2 are the dissociation constants of the inactive complexes E_1G and E_2G, respectively. The over-all rate of the reaction is measured by the formation of P, *i.e.*, k_4 $[I_2]$ $[I_3]$.

If a steady state is established, we may write

$$\begin{cases} \dfrac{d[I_1]}{dt} = k_1[E_1][S] - (k_2[E_2] + k_3)\ [I_1] = 0 \\ \dfrac{d[I_2]}{dt} = k_2[E_2][I_1] - k_4[I_2][I_3] = 0 \\ \dfrac{d[I_3]}{dt} = k_3[I_1] - k_4[I_2][I_3] - k_5[I_3] = 0 \end{cases}$$

Assuming that the concentration of substrate ([S]) remains constant, these equations lead to the following formula:

$$H = \frac{\phi_2[G]^2 + \phi_1[G]}{\phi_2[G]^2 + \phi_1[G] + \phi_0} \qquad (4)$$

where $\phi_0 = K_1K_2(k_2\varepsilon_2 + k_3)$, $\phi_1 = K_1k_3 + K_2k_3 + K_2k_2\varepsilon_2$, $\phi_2 = k_3$, and ε_1 and ε_2 are the total concentrations of enzymes E_1 and E_2, respectively.

Eq. (4) represents the formula for the "intermediate type" of inhibition, and it turns into the formula for the second order inhibition when $\phi_2[G]$ is sufficiently large compared with ϕ_1. The reaction such as represented by the above schema may occur abundantly in the mechanism of photosynthesis. It is also highly probable that in the sequence of reaction steps involved in the dark reaction, two or more steps are catalyzed by one and the same kind of enzyme. At any rate, the example given above may suffice to account for the possibility that certain substances may cause a second order inhibition of the over-all reaction, while they inhibit each intermediate step in the manner of the first order. The fact that azide causes inhibitions of different orders upon photosynthesis and the Hill reaction cannot, therefore, militate against the argument that the latter represents an intermediate step in the former.

SUMMARY

1. Using intact cells of *Chlorella ellipsoidea* (and partly the grana of spinach leaves), the three O_2-liberating reactions shown by plant cells, namely, photosynthesis, the Hill reaction and the catalatic H_2O_2-decomposition, were compared in respect to their susceptibility towards the inhibitors, hydroxylamine, azide, cyanide and 2,4-dinitrophenol.

2. It was found that hydroxylamine and azide inhibit all three reactions, cyanide inhibits photosynthesis and catalatic H_2O_2-decomposition, but not the Hill reaction, while 2,4-dinitrophenol inhibits photosynthesis and the Hill reaction, but not the catalatic H_2O_2-decomposition.

3. Inhibition of photosynthesis by cyanide is of the "first order" while hydroxylamine, azide and 2,4-dinitrophenol cause "second order" inhibition of photosynthesis. On the other hand, the inhibition of the Hill reaction by hydroxylamine and 2,4-dinitrophenol is of the second order, while it is inhibited by azide in the manner of the first order. Inhibition of the catalatic H_2O_2-decomposition by hydroxylamine, azide and cyanide is equally the first order.

4. A reaction schema was proposed, in which an inhibitor may cause a second order inhibition of the over-all reaction, as a result of first order inhibition of some of the intermediate steps involved. It was discussed that the evolution of oxygen by the Hill reaction is accomplished by the same mechanism as in ordinary photosynthesis, and that, on the other hand, the insensibility of the Hill reaction towards cyanide speaks strongly against the theory that catalase participates in the mechanism of photosynthesis.

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STUDIES ON XANTHURENIC ACID

IX. ON THE CONJUGATED COMPOUNDS OF XANTHURENIC ACID IN THE BODY

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Musajo (*1*) succeeded in isolating xanthurenic acid from the urine of white rats in 1935. Later Lepkovsky *et al.* (*2*) proved it to be an abnormal metabolite of tryptophan produced by pyridoxine deficiency. Kotake and Inada (*3, 4*) re-examined it furthermore and established the fact that by administering fairly large doses of both tryptophan and sodium salt of fatty acid simultaneously to white rats, enough of it could be produced in their bodies as to cause some toxic effects such as diabetic symptoms. But this effect is not always apparent by the presence of xanthurenicase in animal, the details of which have been already reported by Kotake, Kotake and Inoue (*5*). As another means of such a detoxication we found in the recently conducted experiment that a part of xanthurenic acid injected into the bodies of normally healthy white rats could be conjugated to some non-toxic compounds and excreted into urine.

Delgliesh, Knox and Neuberger (1951) (*6*) Dalgliesh (1952) (*7*) reported already that when they administered xanthurenic acid to white rats of pyridoxine deficiency and examined the urine and its extract by means of paper partition chromatography, and absorption spectrum, they could detect a spot which indicated the presence of a conjugated xanthurenic acid. Nothing definite, however, has been clarified about it.

EXPERIMENTAL

After feeding more than 2 weeks on synthetic diet, each rat was put in a metabolism cage and injected hypodermically with ammoniacal solution of xanthurenic acid (m.p. 296.5°) adjusted to pH 7.2–7.4. The 24 hour urine was collected and examined.

Quantitative Analysis

Xanthurenic acid in the urine was quantitatively analyzed according to Glazer and Müller's method (*8*) before and after hydrolysis with 1–3 *N* HCl at 100° for 1 hour, the hydrolyzate was cooled, neutralized with sodium carbonate, and then submitted to a quantitative analysis of xanthurenic acid. The results are shown in Table I.

TABLE I

	Body weight	Xanthurenic acid injected	Amount of xanthurenic acid in urine	
		(*100 mg./dl.*)	Before hydrolysis	After hydrolysis
	g.	*mg.*	*mg.*	*mg.*
A	230	23	14.47	17.14
B	230	23	13.18	20.39
C	200	20	15.15	18.21
D	200	20	14.64	18.82
E	190	19	15.28	18.01

As shown in Table I the amount of free xanthurenic acid increased after the hydrolysis: the urine should have contained some conjugated compounds of xanthurenic acid. The 24 hour urine of normal healthy white rats was proved to contain no xanthurenic acid even after hydrolysis.

Paper Partition Chromatography and Absorption Spectrum

Method—One-dimensional and ascending method.

Filter Paper—Toyo filter paper, No. 52.

Developer—*n*-Butanol-acetic acid-water (4 :1 :1) (BAW), and methanol-*n*-buthanol-benzene-water (2 : 1 : 1 : 1) (MBBW).

Developing Time—12 hours.

The 24 hour urine collected from each animal injected with synthetic xanthurenic acid, was mixed with an equal amount of ethyl alcohol, left overnight in an ice-box, and then filtered. The filtrate was concentrated at reduced pressure and submitted to chromatographic analysis.

As shown in Fig. 1, four spots were obtained: F_1 and F_2 appeared closely to each other in case of BAW developing, and F_3 and F_4 which were found partly overlapping with use of BAW were distinctly separated in the case of MBBW. In order to get unified substance as possible MBBW was used as a developer and each spot was extracted with neutral phosphate buffer solution.

Each extract was concentrated at reduced pressure and re-developed with BAW. The spots thus obtained were extracted again and the extracts were examined. The results are given in Table II.

Fig. 1

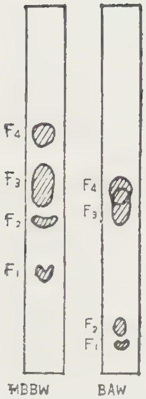
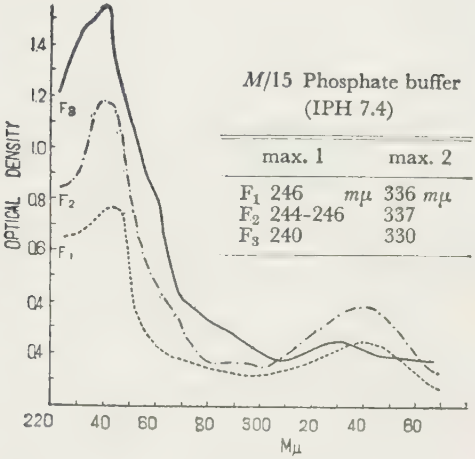


Fig. 2



M/15 Phosphate buffer (IPH 7.4)

	max. 1	max. 2
F_1	246 *mμ*	336 *mμ*
F_2	244-246	337
F_3	240	330

Table II

		F_1	F_2	F_3	F_4	Xanthurenic Acid
Rf. value	M.B.B.W.	0.28	0.42	0.52	0.65	0.52
	B.A.W.	0.08	0.13	0.45	0.47	0.45
Fluorescence		Dark purplish blue	Dark bluish purple	Pale blackish purple	Pale blue	Pale blackish purple
Pauly's diazo reaction		—	Orange red reaction somewhat retarded	Red	—	Red
Millon		—	Orange	Orange	Red reaction retarded	Orange
Fe^{+++}		—	Green	Green	—	Green
Ammoniacal silver reaction*		—	+ Reaction retarded	+	+ Reaction retarded	+
Ninhydrin		—	—	—	—	—

* Heated at 100° after spraying ammoniacal silver reagent.

As shown in Table II both F_2 and F_3 gave xanthurenic acid color reactions such as Pauly's diazo, Minnon's, Fe^{+++} and ammoniacal silver reactions, while F_1 revealed no such reaction. The substance represented by F_4 was so small in amount that its reactions were very weak.

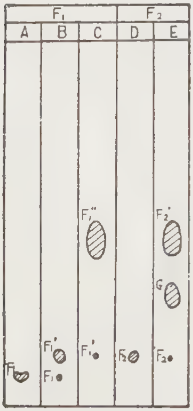
On the same extracts, absorption spectrum was measured by using Beckman spectrophotometer. The results are illustrated in Fig. 2.

Xanthurenic acid generally has two peaks at 240 and 330 mμ. The peaks of F_3 were quite the same, while those of F_1 and F_2 were nearly but not completely similar. As for the substance represented by F_4 it was too small in quantity to permit spectrum study.

But this F_4 spot could be detected in normal rat urine, and thus it seems quite unlikely that F_4 is some conjugated compound of xanthurenic acid.

Next, the F_1 extract was hydrolyzed with 0.1 *N* HCl in a sealed tube at 100°, 1 hour. The hydrolyzate showed on paper partition chromatogram a new spot F_1' (Rf 0.14) of the same fluorescent color, just above the original spot F_1 (Rf 0.08) as shown in Fig. 3; some qualitative tests on both of them were indicated in Table III.

Fig. 3. Developer: BAW



A Before hydrolysis
B After hydrolysis (0.1 *N* HCl)
C After hydrolysis (1,3,5-*N* HCl)
D Before hydrolysis
E After hydrolysis (0.1 *N* HCl)

When more concentrated HCl (1–5 *N*) was used for hydrolysis, however, spot F_1 could no longer be seen, but instead, F_1' (Rf 0.14) and F_1'', a new spot of positive diazo reaction of the latter was the same with xanthurenic acid (Rf 0.45).

This spot F_1'' became more intensified, while F_1' (Rf 0.14) more faded as the hydrolysis proceeded. The fact might be interpreted by assuming that the substance represented by F_1 is a compound conjugated in two position at 4 and 8 of quinoline ring and that the linkage at 4 is easily attacked by 0.1 *N* HCl, but that at 8 can only be attacked by more concentrated HCl.

From the extract of spot F_1'' crystalline substance could be obtained, which seems to be a conjugated compound of

Table III

	F_1 A, B, C.			F_2, E			Glucuronic acid
	F_1	F_1'	F_1''	F_2	G	F_2'	
Rf. value	0.08	0.14	0.45	0.13	0.30	0.45	0.30
Fluorescence	Dark purplish blue	Dark purplish blue	Pale blackish purple	Pale bluish purple	—	Pale blackish purple	—
Pauly's diazo reaction	—	—	+	±	—	+	—
Ammoniacal silver reaction				—	+	+	+

xanthurenic acid with some ether-sulfate compound. The ultimate nature of this compounds awaits further study.

Next, the extract of F_2 was treated with 0.1 N HCl in the same way as above. Spot F_2'' of positive diazo reaction appeared this time at the position of xanthurenic acid (Rf 0.45), and another spot of positive ammoniacal silver reaction at that of Rf 0.3, while spot F_2 (Rf 0.13) vanished almost entirely. On examining by Beckman spectrophotometer, it was found that, as shown in Table IV, both F_1'' and F_2' furnished quite the same absorption spectrum with free xanthurenic acid.

Furthermore, in the hydrolyzate of F_2 there existed a substance, which gave the orcin color reaction of glucuronic acid. This finding leads us to believe that F_2 is due to a glucuronide of xanthurenic acid and that the conjugation occurred through OH radical at the position 4, since its diazo and the like other reactions were positive.

Table IV

(M/15 phosphate buffer at pH 7.4)

Substance	Position of 1st max.	Position of 2nd max.
M/20000 xanthurenic acid	240 mμ	330 mμ
Extract of B, F_1'	240	336
Extract of C, F_1''	240	330
Extract of B, E_2	240	330

SUMMARY

From the experimental results so far obtained the following conclusions may be made.

1. From the urine of rats injected with xanthurenic acid, two kinds of conjugated compounds were obtained.

2. One of them gave positive diazo reaction by itself, could easily be hydrolyzed by 0.1 *N* HCl and its hydrolysate presented positive orcin reaction of glucuronic acid. The substance is most likely a glucuronide of xanthurenic acid, the conjugation being occurred at OH radical at the position 4.

3. Another one gave negative diazo reaction by itself, could only be hydrolyzed by concentrated HCl. In this case the conjugation might have occurred at the position 8 of xanthurenic acid with some ether-sulphate compound. In all probability this free OH radical is diabetogenic agent for rat.

We heartily thank Prof. Yashiro Kotake for his pertinent advices, encouragements and his revision and also heartily Prof. Keizo Kodama for his revision to this study.

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STUDIES ON XANTHURENIC ACID

X. PROGRESSIVE DEPLETION IN THE REDUCED GLUTATHIONE CONTENT OF THE BLOOD FOLLOWING XANTHURENIC ACID INJECTION

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The close relationship between diabetogenic substances and reduced glutathione (GSH) has been studied by many research workers. Leech and Baily (*1*), among others, examined the effects of alloxan in 1954 and observed a remarkable decrease of the blood GSH content by alloxan injection.

In this line it seems to be interesting to investigate the effect of xanthurenic acid, which was found by Kotake, Jr. and Inada (*2*) to be a diabetogenic substance, upon blood GSH content. The results of such an investigation are here reported.

METHOD

Male white rats weighing about 150 g. were fed over week on the diet conposed of 22 per cent casein, 52 per cent starch, 2 per cent yeats, 3 per cent agar, 6 per cent McCollum salt mixture, 10 per cent butter and 5 per cent sucrose.

To one group of these white rats 200 mg. of xanthurenic acid were injected into intraperitoneal cavity, and blood sugar was determined every thirty minutes after the injection. At 3–4 hours after the injection when the initial hyperglycemia period began, the animals were sacrificed by cutting carotid artery, and the blood was collected for the examinations.

For the sake of comparison, untreated white rats and those received epinephrine injection were the victims, under the same feeding condition.

GSH content was determined by the iodine titration method improved by Fujita and Iwatake (*3*) on the Tunnicliffe's (*4*) by use of metaphosphate to eliminate protein.

The blood sugar of each rats was determined by King and Garner's method (*5*) on 0.02 ml. of blood taken from a vein of the tail.

RESULTS

The results are shown in Table I. The mean value of the blood GSH content of seven normal healthy rats was 30.3 mg./dl., while in case of the rats injected with xanthurenic acid, the mean value was as low as 15.2 mg./dl., the lowest value being 7.6 mg./dl.

TABLE I

The Effects of Xanthurenic Acid on Blood GSH Contents and Blood Sugar Amount

I	Untreated rat		II	Xanthurenic acid-injected rat		III	Epinephrine-injected rat	
No.	Blood GSH *mg./dl.*	Blood sugar *mg./dl.*	No.	Blood GSH *mg./dl.*	Blood sugar *mg./dl.*	No.	Blood GSH *mg./dl.*	Blood sugar *mg./dl.*
1.	32.5	82.7	8.	24.5	134.3	15.	29.4	224.6
2.	32.2	69.0	9.	20.5	156.8	16.	32.5	275.0
3.	29.5	73.4	10.	12.3	118.6	17.	30.0	280.0
4.	29.5		11.	15.3		18.	36.3	188.6
5.	26.0	74.8	12.	16.9		19.	27.0	302.0
6.	32.5		13.	9.2				
7.	30.0		14.	7.6				
Mean value	30.3			15.2			31.0	

The state of this low blood GSH content continued fairly long time. The dyas after the injection of 200 mg./kg. of xanthurenic acid, for instance, to a male rat weighing 160 g., its blood sugar remained still as high as 120.8mg./dl., while its GSH content of blood was only 16.8 mg./dl. The latter value is nearly the same, as shown in the above table, as those found after 3–4 hours of xanthurenic acid injection.

It is noteworthy that hyperglycemia produced by epinephrine was not accompanied by such a lowering of the blood GSH contents. It can be concluded, thereafter, that the decrease of blood GSH is caused by specific action of xanthurenic acid.

By way of a further experiment several rabbits fed on vegetable and soaked bean cake ("Okara") were employed to investigate the effect of the administration of xanthurenic acid. Approximately identical results were obtained as in the previous experiment with albino rats. (*cf.* Table III).

Shown below are the values of blood GSH content of normal healthy persons and of considerably serious diabetic patients. As shown in Table II, the blood GSH content of diabetic patients was found to be decreased almost by half to that of normal healty persons. The same tendency observed between animal and human cases may be of great interest.

TABLE II

The Relationship between Diabetic Patients and Normal Healthy Persons on Blood GSH Contents and Blood Sugar Amount

No.	Blood GSH *mg./dl.*	Blood sugar *mg./dl.*	No.	Blood GSH *mg./dl.*	Blood sugar *mg./dl.*
1	16.8	760.8	1	27.6	64.7
2	16.8	417.6	2	26.9	82.3
3	16.9	672.3	3	24.6	75.0
4	19.9	229.0			
5	15.3	257.6			
Average	17.1	467.4	Average	27.0	74.0

TABLE III

The Blood GSH Contents of Rabbits after the Injection of 59 mg./kg. Xanthurenic Acid

No.	Before injection *mg./dl.*	After injection *mg./dl.*
1	32.2	20.0
2	30.7	18.5
3	33.5	21.7
Average	32.1	20.1

SUMMARY

Using rats, it has been proved that an injection of 200 mg./kg. of xanthurenic acid leads to an unmistakable decrease of blood GSH.

In conclusion, we heartily thank Prof. Yashiro Kotake for his pertinent advices, encouragements and his revision. We also heartily thank Prof. Keizo Kodama for his revision to this study.

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RESPIRATORY OXIDATION AND OXIDATIVE PHOSPHORYLATION BY CYTOPLASMIC PARTICLES OF SWEET POTATO

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(Received for publication, June 30, 1954)

Demonstration of oxidative phosphorylation in higher plant tissues such as mung bean has shed the light on the mechanism of respiratory system of plants. This is due to the excellent works of Bonner's laboratory (*1*), and similar results were achieved by Maruo *et al.* (*2*) independently. Mung bean seedlings which have higher metabolic activity are suitable source for the researches of plant respiration, and many workers have used them; for example, the above authors succeeded first in the proof of oxidative phosphorylation using the cytoplasmic particles of them, and Albaum *et al.* (*3*) extracted highly purified ATP from them. The cytoplasmic particles prepared from sweet potato, also, were found to carry out not only the oxidation of members of tricarboxylic acid (TCA) cycle, but the active phosphorylation coupled with the oxidation. It has been understood that the oxidative and phosphorylative activities of the cytoplasmic particles of sweet potato are similar to those of mung bean tissues.

EXPERIMENTAL

Methods and Materials

The method of the enzyme preparation was shown in Fig. 1. It was similar to that of Bonner's method (*1*) except a few improvements. In sweet potato tissues, there were found the higher activity of adenosine triphosphatase (ATP-ase) and large amount of Ca ion which stimulated ATP-ase. As ATP-ase depresses the oxidative phosphorylation by the particles, it must be made inactive in the particles. Therefore NaF to inhibit ATP-ase and versene (sodium ethylenediaminetetraacetate) to trap Ca ion were added to the sucrose solution to prepare the enzyme (particles suspension).

Ingredients of the reaction mixture to determine the oxidation of members of TCA cycle by the particles of sweet potato were shown in Table II. Oxygen uptake was measured for 60 minutes at 30° using the Warburg's manometric method.

TABLE I

Outline of Procedures Used in Preparation of Particles from Sweet Potato

Sweet potato root
—Peal and slice

80 g. of tissue and 70 ml. of 0.5 *M* sucrose solution containing 0.1 *M* phosphate buffer (pH 7.2) and 0.2 *M* NaF
—Blend with Waring blendor, 1/2 minutes at 0–6°
—Filter through gauze

Light yellow brown suspension
—Adjust to pH 7.2–7.4
—Centrifuge at 1000×*g*, 5 minutes at 0–6°

Orange brown supernatant
—Centrifuge at 15,000×g, 20 minutes at 0°

Precitate
(unbroken cell, walls and starch)

Cytoplasmic particles
—Resuspend in 10 ml. of 0.5 *M* sucrose, containing 0.1 *M* phosphate buffer (pH 7.2), 0.2 *M* NaF and 0.01 *M* versene
—Adjust to pH 7.2–7.4
—Centrifuge at 1,000×*g*, 5 minutes

Supernatant contg. submicroscopic particles

Cytoplasmic suspension
—Centrifuge at 15,000×*g*, 20 minutes at 0°

Precipitate
(Starch)

Cytoplasmic particles
(presumably mitochondria)
—Take up 5 ml. of 0.5 *M* sucrose solution containing 0.02 *M* phosphate buffer (pH 7.2), 0.2 *M* NaF and 0.01 *M* versene
—Adjust to pH 7.2–7.4

Supernatant

Particulate enzyme preparation

The reaction mixture to determine the phosphorylation by the particles contained such ingredients as shown in Table IV. Oxygen uptake for 40 minutes was measured at 30°. Phosphate uptake was measured as follows: At zero time (initial) 1ml. -aliquot of reaction mixture was removed from one vessel, put into 1 ml of 10 per cent trichloroacetic acid solution and centrifuged; then inorganic phosphate in the supernatant was determined by Allen's (*4*) method modified by Nakamura (*5*). After 40 minutes of incubation at 30°, 1 ml. -aliquot of reaction mixture was removed from each vessel and analyzed for inorganic phosphate by above mentioned procedure. A mark P/O shown in Tables means the ratio of inorganic phosphate uptake to oxygen uptake in μ atoms per 1 ml. reaction mixture.

Table II

Oxidation of Members of TCA Cycle by Sweet Potato Partiles

The control system contained 50 μM of phosphate buffer (pH 7.2), 250 μM of sucrose, 3 μM of ATP, 10 μM of $MgCl_2$ and 1.0 ml. of enzyme in a total volume 2.5 ml. 60 minutes incubation, at 30° in Warburg vessels.

Substrate	Concn. (μM)	Oxygen uptake μM/ml./60 min.
None	0	0
Citrate	20	1.07
α-Ketoglutarate	20	1.20
Succinate	20	1.29
Malate*	20	0.68

*Separate but comparable experiment.

Results

As shown in Table II, the particles of sweet potato root oxidized the members of TCA cycle; citrate, α-ketoglutarate, succinate and malate respectively. In repeated experiments, without exception, the oxidation of succinate was highest and that of malate was lowest. The results were fairy good agreement with those of Bonner and Maruo who used mung bean mitochondria.

Generally pyruvate oxidation by cyclophorase system is promoted by addition of catalytic amount of a member of TCA cycle such as malate or succinate. Proof of this phenomenon, so-called sparking effect, may give one cogent evidence for the presence of TCA cycle in the tissues. Table III shows that the oxygen uptake of combined

Table III

Sparking Effect of Malate on Pyruvate Oxidation

(Addenda as for Table II)

Substrate	Concn. (μM)	Oxygen uptake μM/ml./60 min.
None	0	0
Pyruvate	25	0.03
Malate	1	0
Pyruvate+malate	25 & 1, resp.	0.59

system, pyruvate (25 μM)+malate (1 μM), was very large; but with either alone it was very small. TCA cycle was supposed to be oparative in the particles of sweet potato also.

Coupling of phosphorylation by the particles to aerobic oxidation of members of TCA cycle was established as shown in Table IV. As above-mentioned, in order to inhibit the higher activity of ATP-ase contained in sweet potato root, NaF was added to the sucrose solution to prepare the enzyme (particles suspension). The influence of further addition of NaF was tested on the oxidative phosphorylation by the enzyme preparation. As shown in Table IV, addition of 20 μM of NaF had no influence on the phosphorylation, but that of 100 μM exerted an unfavourable effect.

Table IV

Oxidative Phosphorylation by Sweet Potato Particles

The system contained 20 μM of phosphate buffer (pH 7.2), 200 μM of sucrose, 20 μM of one of the TCA cycle members, 3 μM of ATP, 10 μM of $MgCl_2$, 100 μM of glucose, 10 μM of NaF, 10 μM of versene, 3 mg. of the hexokinase and 1 ml. of enzyme in a total volume of 2.5 ml. The hexokinase was prepared from yeast by the method of Berger. (*8*) 40 min. incubation, at 30° in Warburg vessels.

Substrate	Oxygen uptake*	Phosphate uptake*	P/O
Citrate	2.29	2.6	1.13
α-Ketoglutarate	2.35	2.35	1.0
Succinate	2.5	2.6	1.04

*Uptake in μ atoms/ml./40 min.

Table V

Influence of NaF on the Oxidative Phosphorylation by Sweet Potato Particles

(Addenda as for Table IV)

Substrate	Concn. of NaF	Oxygen uptake*	Phosphate uptake*	P/O
	μM			
Citrate	0	2.47	2.48	1.0
Citrate	20	2.4	2.36	0.98
Citrate	100	1.73	1.32	1.31

*Uptake in μ atoms/ml./40 min.

From the biochemical point of view, it is very interesting to know whether or not oxidation of polyphenol by polyphenol oxidase is coupled with generation of high energy phosphate bond. Further, it is also significant to elucidate what influence the oxidation of chlorogenic acid, a polyphenol in sweet potato, has on the phosphorylation coupled with oxidation of a member of TCA cycle.

Goddard *et al.* (*6*) reported that polyphenol oxidase in white potato was collected in the supernatant by high speed centrifuge separation, but McClendon (*7*) clarified that polyphenol oxidase was present in all fractions in quantities roughly proportional to the protein content. We confirmed, already, highly activated polyphenol oxidase was contained in the cytoplasmic particles of sweet potato.

As the cytoplasmic particles contained polyphenol oxidase as well as phosphorylation enzyme system, they were supposed to be adequate as the enzyme preparation to solve the above question. As shown in Table VI the oxidation of chlorogenic acid by the particles was strong, but the concomitant oxidative phosphorylation was not observed. Seemingly the oxidation of chlorogenic acid itself did not produce high energy phosphate bond. The coupling of phosphorylation to citrate oxidation was depressed in the presence of chlorogenic acid; the inhibition seemed to be due to the quinone produced by the oxidation of chlorogenic acid, as the inhibitory effect of chlorogenic acid was not observed on the oxidative phosphorylation by the particles of mung bean that were free from polyphenol oxidase activity.

TABLE VI

Influence of Chlorogenic Acid Oxidation on Oxidative Phosphorylation Coupled with Citrate Oxidation

(Addenda as for Table IV, except for 60 minutes incubation.)

Substrate & concn.	Oxygen uptake*	Phosphate uptake*	P/O
Citrate, 20 μM	2.37	2.96	1.25
Citrate, 20 μM + chlorogenic acid 2 μM	4.27	1.78	0.42
Chlorogenic acid 2 μM**	1.42	0	0

* μ atoms/ml./60 min. **Separate but comparable experiment.

DISCUSSION

The cytoplasmic particles prepared from sweet potato were found to carry out not only the oxidation of members of TCA cycle, but the active phosphorylation coupled with the oxidation. It is described briefly in Stumpf's (*9*) paper that Saltman observed the complete oxidation of intermediates of TCA cycle by the particles of sweet potato, but the details are obscure. We (*10*) canfirmed that cytochrome oxidase was contained in the particles of sweet potato and it took part in respiration actually (*11*). Webster (*12*) observed independently the fact. And Saltman (*13*) reported hekokinase was involved in the particles of sweet potato and other higher plants. These facts indicate that the respiratory mechanism of sweet potato tissue is much the same as that of other plants.

It was understood that the phosphorylation by the particles of sweet potato, coupled with citrate oxidation, was prevented in the presence of chlorogenic acid, and the inhibition might be caused by the quinone, an oxidative product of chlorogenic acid. This is similar to the result obtained by Friedkin and Lehninger (*14*) that hydroquinone caused inhibition of phosphate esterification during malate oxidation. Seemingly the oxidation of chlorogenic acid itself did not generate the high energy phosphate bond. The phenomenon may be explained by either of the two assumptions: 1) the oxidation of chlorogenic acid is really uncoupled with phosphorylation, and 2) the phosphorylative reaction is inhibited by the action of quinone produced by chlorogenic acid oxidation. However, we can hardly suppose the possibility of latter case, because the phosphorylation coupled with citrate oxidation was not completely inhibited in the presence of chlorogenic acid. Presumably the oxidation of chlorogenic acid may be actually uncoupled with phosphorylation.

We have been studying the mechanism of respiratory increase in black-rotten sweet potato. P.J. Allen (*15*) presumed from his observation of inorganic phosphate increase and Pasteur effect inhibition in wheat, infected by powdery mildew, the respiratory increase should be explained by that the mildew toxines acted as uncouplers and accelerated the release of inorganic phosphate and regeneration of ADP; thus a given level of phosphate acceptors wouled lead to a more rapid oxidation. We had an assumption if something metabolized in the infected sweet potato might play a role as uncouplers and permit the respiratory increase, and observed that ipomeamarone (*16*, *17*), an abnormal metabolite accumulated in the infected tissues, stimulated not only the respiration of sweet potato slices, but exerted uncoupling effect on the oxidative phosphorylation carried out by the cytoplasmic particles of mung bean and sweet potato (*18*). Thus, ipomeamarone was supposed to be a factor of the respiratory increase. Both cytochrome oxidase and polyphenol oxidase were much more activated in black-rotten sweet potato than in sound one (*10*), and it was supposed from the experiment of CO-inhibition and its photoreversibility (*11*) that both cytochrome oxidase and polyphenol oxidase would take part in the respiration of black-rotten sweet potato, but cytochrome oxidase alone would be the actual terminal oxidase of sound one. As polyphenol oxidase was supposed to have no relation to phosphorylation cycle which might regulate the rate of respiration, participation of polyphenol oxidase system in respiration also would be considered as a factor of the respiratory increase in the rotten sweet potato.

In both animal and plant tissues, the metabolic functions of cytoplasmic particles, mitochondria, have become uncovered by many workers (*1*, *2*, *21*). Elucidation of phosphorylative activity of the cytoplasmic particles is the most striking result, and some energy requiring reactions such as glutamine synthesis (*22*) or glutathione synthesis (*23*) have also been observed. We wish to establish much more conclusive reaction sequence in the particles of sweet potato.

SUMMARY

1. The cytoplasmic particles prepared from sweet potato were found to carry out the oxidation of members of TCA cycle, and promote the pyruvate oxidation in the presence of catalytic amount of one member of TCA cycle; therefore TCA cycle was supposed to be oparative in the particles of sweet potato.

2. The cytoplasmic particles of sweet potato were proved to be capable of generating high energy phosphate bond under coupling with respiratory oxidation of members of TCA cycle.

3. The phosphorylation by the particles of sweet potato, coupled with citrate oxidation, was prevented in the presence of chlorogenic acid. The inhibition was supposed to be ascribed to quinone produced through oxidation of chlorogenic acid by polyphenol oxidase in the particles.

4. The oxidation of chlorogenic acid itself by polyphenol oxidase in the particles did not generate the high energy phosphate bond and the oxidation was understood to be actually uncoupled with phosphorylation.

We are grateful to Prof. Saburo Funahashi, Nagoya University, for his cordial advice and encouragement.

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OXIDATION REDUCTION ENZYMES OF A HALOPHILIC BACTERIUM

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There are possibly two points of view to explain the mechanism causing halophilism to appear in halophilic bacteria. One is that the halophilism is based upon the expenditure of the metabolic energy necessary to maintain the salt concentration within the cells lower (J. Robinson *et al.* (*1*)). The other is that these organisms contain particular enzymes called by the author (*2*) "halophilic enzymes" which require for their action a rather high salt concentration of the media as demonstrated in halophilic alkaline phosphomonoesterase. The present paper deals with the investigations on oxidation reduction enzymes of the halophilic bacterium. Examination in respect of halophilism of glucose dehydrogenase and formic dehydrogenase, both of which had been obtained in a cell-free state, showed that only glucose dehydrogenase was halophilic.

EXPERIMENTAL

Bacterium—The test microorganism isolated by H. Yamada was species of *Pseudomonas*, tentatively called No. 101. *Ps. fluorescens* and *E. coli* were used as control organisms.

Preparation of Enzyme Solution—The halophilic bacteria were cultured and harvested as previously described (*2*). Gathered cells were suspended in distilled water and were exposed to the ultrasonic wave (480 K. C.) for 30 minutes and then centrifuged at $18,000 \times g$ for 20 minutes. The yellow supernatant thus obtained was used as enzyme solution. The enzyme of *Ps. fluorescens* was obtained by using the ultrasonic vibration from cells ordinarily cultured. Formic dehydrogenase of *E. coli* was prepared in the same way as with the nitrate reductase reported by Egami and Sato (*3*).

Measurement of Activity—O_2-uptake was measured by Warburg's manometer at 37°, pH 6.8; 0.3 ml. enzyme solution, 0.3 ml. substrate ($M/10$), 0.4 ml. phosphate buffer ($M/4$), and $5M$ NaCl solution and/or distilled water were added to the main chamber to make a total volume of 3 ml. and 0.1 ml. NaOH (10 per cent) was added to the central well. The gaseous phase was left as air. The activities of dehydrogenases were determined by the Thunberg technique and expressed by the reciprocal values of decolorization time at 37°, pH 6.8. The main tube contained 1 or 0.5 ml. enzyme solution, 0.5 ml. phosphate buffer ($M/2$), and $5M$ NaCl solution and/or distilled water. The side arm contained 0.5 ml. substrate ($M/10$). Total volume of contents was 5 ml. In measuring the activities of glucose dehydrogenases toluylene blue was chosen as the most suitable dye-stuff while methylene blue was used for formic dehydrogenases.

RESULTS

Oxidation of Glucose—The extract of the halophilic bacteria contained an enzyme system by which the oxygen absorption for glucose increased almost linearly with time, irrespective of the amounts of NaCl added as shown in Fig. 1. It might be seen from Fig. 2 that for each mole of glucose consumed one gram atom of oxygen was absorbed.

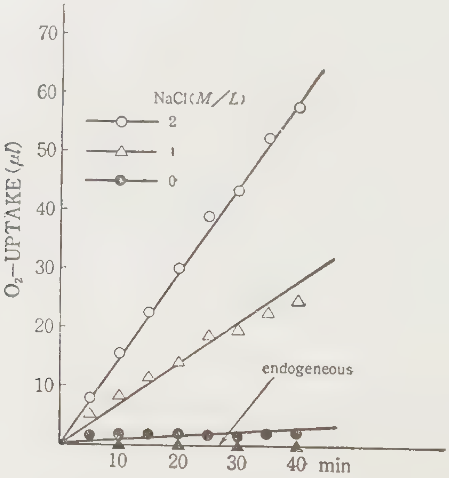

FIG. 1. Glucose oxidation by the cell-free extract of the halophilic bacterium.

The fact that this oxidation of glucose was not inhibited by iodoacetate but notably by cyanide and azide, as shown in Table I, suggested that the O_2-uptake system contained a cytochrome system in addition to glucose dehydrogenase. The effects of various salts on the oxygen uptake were examined and are illustrated in Fig. 3, in which relative values of initial velocity were plotted against cation concentration with the maximum rate for NaCl as 100 of the ordinate scale. When using sodium citrate, the pH of the test solution was adjusted to 6.8 by HCl. It may be pointed out from Fig. 3 that activity-concentration curves take characteristic shapes

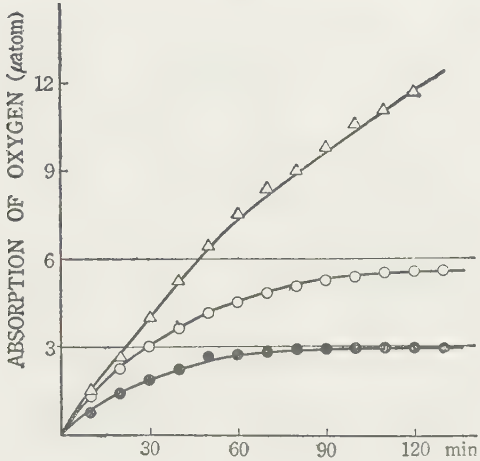

FIG. 2. Ratio of oxygen absorbed to glucose consumed. Horizontal lines represent theoretical values of oxygen calculated with the ratio as unity.

Amounts of glucose added as follows;
—△— 60 μM, —○— 6 μM, —●— 3 μM.

TABLE I

Inhibitory Effects of Various Chemicals on O_2-uptake with Glucose by the Extract from the Halophilic Bacterium

Inhibitor / Concentration	Degree of inhibition (%)			
	KCN	NaN_3	NaF	Iodoacetate
10^{-1} *M*			87	
3×10^{-2}			60	
2×10^{-3}	70	84		
10^{-3}	70	60		0
3×10^{-4}	50	46		

for halophilic enzyme not only in the case of NaCl but in others.

Glucose Dehydrogenase—Because O_2-uptake with glucose appeared to pass through cytochrome system, glucose dehydrogenase activities were measured by the Thunberg technique. Although the dehydrogenase very slowly decolorized methylene

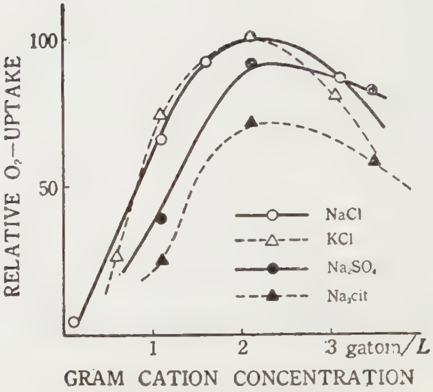

Fig. 3. The effects of various salts on glucose oxidation.

blue, the higher the redox potentials of dyes were the more rapid the rates of decolorization, as already described by Y. Ogura (*4*) with glucose dehydrogenase of *Aspergillus oryzae*. Consequently toluylene blue was employed as test dyestuff also by virtue of its high solubility in a higher salt concentration. The activity-concentration curve for the dehydrogenase plotted similarly as above showed the particular halophilic character without regard to the kinds of salt (Fig. 4) in contrast to a control enzyme extracted from a ordinary strain of *Pseudomonas fluorescens*. When ethylene glycol was used in place of mineral satls as an example of non-electrolytes, this same effect could not be observed.

Formic Dehydrogenase—Formic dehydrogenase could be extracted from the halophilic organism by the same treatment as with glucose dehydrogenase. The decolorization rates by the formic dehydrogenase, however, contrary to the glucose dehydrogenase, decreased with an increase of salt concentration although the degrees of inhibition were lower than in the case of the formic dehydrogenase extracted from a non-halophilic *E. coli* strain. (Fig. 5)

DISCUSSION

As the first example of halophilic oxidation reduction enzymes, the enzyme system related to the oxidation of glucose was extracted from the halophilic bacterium. However, every oxidation reduction enzyme of such organism was not necessarily halophilic. The formic dehydrogenase which was extracted together with the above enzyme system had not the halophilic character. Such a feature is not restricted to oxidation reduction enzyme alone. Hydrolases extracted from the halophilic bacterium are not always halophilic. The only example of halophilic

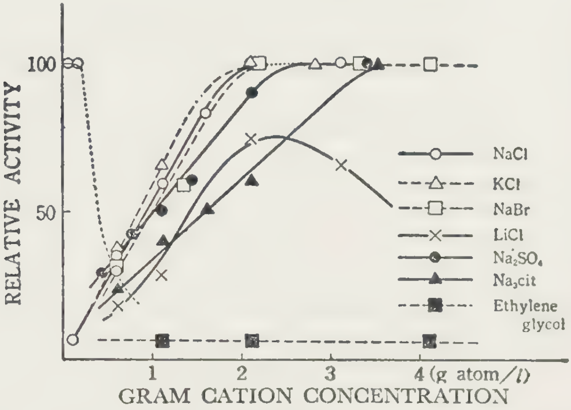

FIG. 4. The effects of various salts on glucose dehydrogenase activity. The unit of abscissa for ethylene glycol is not gram cation but molar concentration.
———, — — — halophilic bacterium. ...... *Ps. fluorescens.*

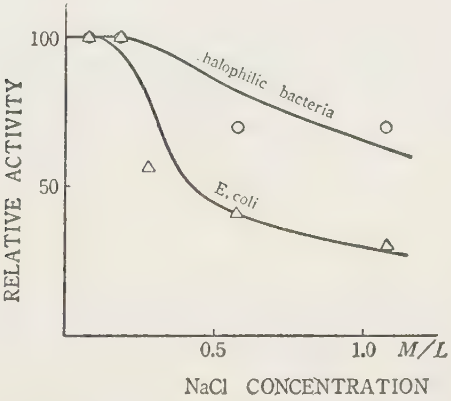

FIG. 5. The effects of NaCl on formic dehydrogenase.

hydrolases so far found is alkaline phosphomonoesterase of this organism (*2*). Therefore, at the present stage of the investigation the halophilism of these bacteria can not be explained alone by the existence of such enzymes within the cells though it may be considered that they have a close relationship to the halophilism.

The characteristic activity-concentration curves can be obtained with various kinds of salt and the shapes of curves could be made similar to each other if the activity is plotted against the gram cation concentration, whereas the factor which determines the O_2-uptake of resting cells of some halotolerant or halophilic bacteria has been shown to be osmotic pressure as revealed by the effectiveness of non-electrolytes (*5*, *6*). It cannot be concluded, however, that the quantitative similarity of shape may be obtained with pure enzymes until these enzymes can be purified to a higher degree.

There are several pathways of glucose oxidation by microorganisms. Inhibitory effects of cyanide and azide on the O_2-uptake with glucose seem to indicate that the oxidation system of this extract contains cytochrome system and excludes all possibilities of the existence of notatin-like enzyme (*7*). Moreover three facts *viz.* that only one gram atom of oxygen was absorbed per mole of glucose oxidized and no carbon dioxide was evolved, and that iodoacetate did not inhibit the oxidation suggest that the pathway of this reaction is not glycolytic but is the alternative of either glucose to gluconate or glucose 6-phosphate to 6-phosphogluconate (*8*, *9*, *10*, *11*) although the oxidation product has not yet been identified. In addition to this, several other points remain unsolved, *i.e.* what kinds of cytochrome system are involved in this oxidation and is only the dehydrogenase halophilic or is cytochrome system also halophilic? These questions arise as it was found that with *p*-phenylenediamine which is a well-known substrate for cytochrome oxidase this extract absorbed oxygen only slowly compared with glucose as substrate.

SUMMARY

1. Glucose oxidizing system, including glucose dehydrogenase, and formic dehydrogenase were both extracted by means of ultrasonic vibration from a halophilic bacterium.

2. The glucose dehydrogenase is halophilic whereas the formic dehydrogenase is not.

3. It is supposed that glucose would be oxidized to gluconate or 6-phosphogluconate and O_2-uptake would pass through cytochrome system by this enzyme system.

4. The mechanism of halophilism was discussed.

The authors are grateful to Professor F. Egami for his kind encouragement and advice. A part of the expense of this research is defrayed by the Scientific Research Encouragement Grant of the Ministry of Education to which the authors' thanks are due.

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The Journal of Biochemistry, Vol. 41, No. 5, 1954

STUDIES ON THE TOXICITY OF FISH OIL. II.*

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(Received for publication, July 15, 1954)

In the foregoing paper, the author reported that ethyl ester of highly unsaturated fatty acid freshly obtained from cuttle fish oil showed quite the same good nutritive effect as oleic acid ester for rat, while the acid exposed to air was proved to be extremely toxic.

Since it seems to be most plausible that the toxicity is concerned with the formation of peroxide by autoxidation of unsaturated fatty acid, the author determined the amount of peroxide. The result proved this was just the case, the details of which will be reported here.

EXPERIMENTAL

The determination of peroxide was carried out following the Wheeler's method improved by Nakamura (*1*). It runs as follows.

An adequate portion of the sample, usually 0.1–0.5 g., was exactly weighed out into conic flask of 300 ml. capacity, mixed with 20 ml. of carbon tetrachloride, 30 ml. of glacial acetic acid and finally 1 ml. of saturated potassium iodide solution. After well shaken for 1 minute, 100 ml. of distilled water was added to and thus free iodine liberated in the solution was titrated by 0.01 *N* thiosulphate solution. 1 ml. of this thiosulphate solution corresponds to 0.00008 g. of oxygen.

The acid ester used here was prepared from cuttle fish oil as described in the foregoing paper and had the following properties.

TABLE I

n_D^{20}	Iodine value. (Wijis)	Sap. value	Unsap. matter %	Peroxide *mg./100 g.*
1.4815	342.1	168.6	Trace	2.

About 50 g. of this ester was poured into Petri-dish and submitted for autoxidation by contact with air at room temperature (2–18°). The determinations of peroxide were carried out at a few days interval over 200 days. The results are illustrated in Fig. 1.

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* The resume of this experiments was reported at the 26th Annual Meeting of the Japanese Biochemical Society, in April, 1954.

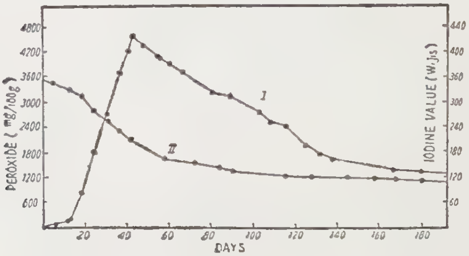


Fig. 1. The change of peroxide (I) and iodine values (II) of the fatty acid ester exposed to air at room temperature (2–18°).

It is interesting to note that during initial 2 weeks the formation of peroxide was almost negligible and then abruptly increased. On the 42nd day, it reached the maximum showing the peroxide value 4400 and iodine value 208. Then it decreased gradually until the peroxide value dropped to 1200, and the iodine value to 120. In the course of this autoxidation the ester became gradually viscous and tinted yellowish brown. On the samples corresponding to various phase of autoxidation, the toxicities were tested by feeding rat with the diet containing 5 per cent of the sample. One which showed the peroxide value 3112 and the iodine value 137 was found fatal to rat, while one with the decreased peroxide value such as 85 and the iodine value such as 76.6 showed no more toxic action.

When the process of autoxidation was allowed to proceed at 30°, the formation of peroxide was accelerated, but its maximal amount was much reduced compared with that at room temperature.

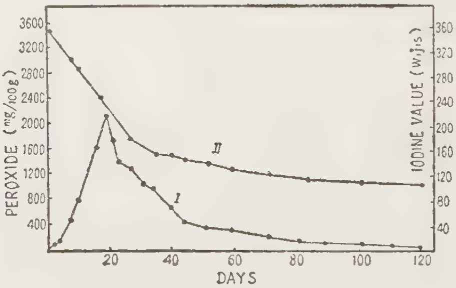


Fig. 2. The change of peroxide (I) and iodine value (II) of the fatty acid ester exposed to air at 30°.

This tendency of the effect of the heat was more apparent at 100°, the maximal amount of peroxide being only 240 in this case. Further it was noticed that at higher temperatures the decomposition of once preformed peroxide occurred more rapidly and thoroughly.

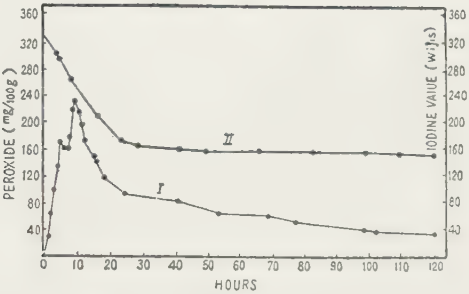


FIG. 3. The change of peroxide (I) and iodine values (II) of the fatty acid ester exposed to air at 100°.

The sample heated at 100° and possessing the peroxide value 240 and the iodine value 245.5 was found fatal for rat, causing abrupt decrease of body weight, which led to death within 7 days, as shown in Fig. 4.

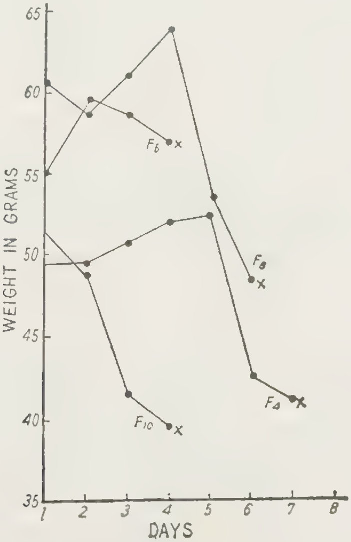


FIG. 4. The effect of the heated fatty acid ester possessing peroxide value of 240 and the iodine value 245.5.

To the contrary, the sample heated further and possessing the peroxide value 30 and the iodine value 156.1 showed no more toxicity. Moreover, its nutritional effect was fairly good as shown in Fig. 5.

This fact suggests an important mean to eliminate the toxicity of fish oil.

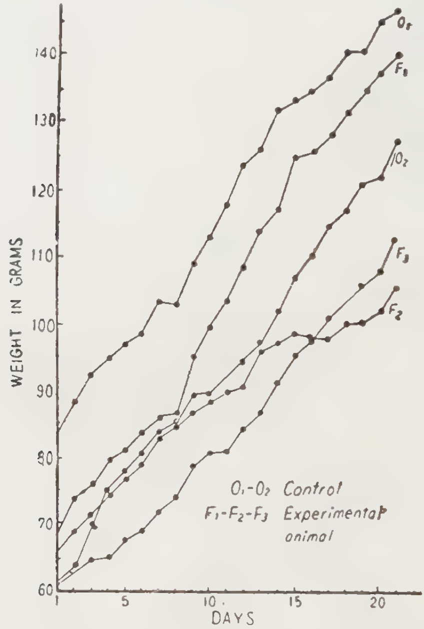
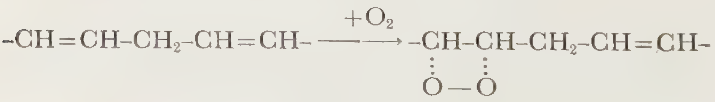


FIG. 5. The growth curve of rat fed on the diet containing 5% of the heated fatty acid ester with the peroxide value 30 and the iodine value 156 in basal diet.

DISCUSSION

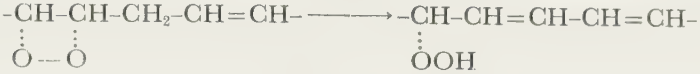
From the experimental results above mentioned it is almost certain that the toxic action of fish oil is due to the peroxide formed by autoxidation at the site of double linkage in the molecule of unsaturated fatty acid.

The chemism involved in peroxide formation is pictured as follows.



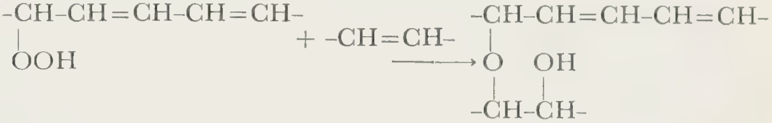
$$\mathrm{-CH{=}CH{-}CH_2{-}CH{=}CH-} \xrightarrow{+\mathrm{O_2}} \underset{\substack{\vdots\qquad\vdots\\ \mathrm{O-O}}}{\mathrm{-CH-CH-}}\mathrm{CH_2{-}CH{=}CH-}$$

According to the recent theory advanced by Gunstone and his co-worker (*2*) as well as by Lundberg and his associates, (*3*) the peroxide radical thus formed has tendency to become hydroperoxide as follows.



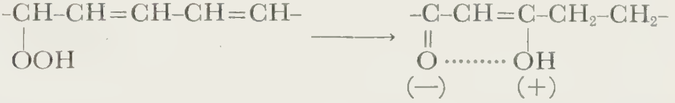
$$\begin{array}{l}\text{-CH-CH-CH}_2\text{-CH=CH-}\\ \vdots\quad\ \ \vdots\\ \text{O}-\text{O}\end{array}\longrightarrow\begin{array}{l}\text{-CH-CH=CH-CH=CH-}\\ \vdots\\ \text{OOH}\end{array}$$

The resulted hydroperoxide compound may easily enter into ether bond with a double bond of other fatty acid molecule in the following way (*4*, *5*, *6*).



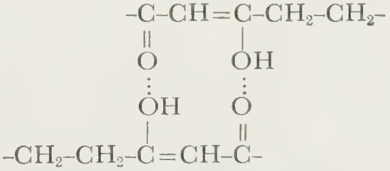
$$\begin{array}{l}\text{-CH-CH=CH-CH=CH-}\\ |\\ \text{OOH}\end{array} + \text{-CH=CH-} \longrightarrow \begin{array}{l}\text{-CH-CH=CH-CH=CH-}\\ |\\ \text{O}\quad\ \text{OH}\\ |\qquad\ |\\ \text{-CH-CH-}\end{array}$$

It is thought also that the presence of –OOH radical activates the double bond close to it, so that the double bond shifts in the following way (*7*).



$$\begin{array}{l}\text{-CH-CH=CH-CH=CH-}\\ |\\ \text{OOH}\end{array}\longrightarrow\begin{array}{l}\text{-C-CH=C-CH}_2\text{-CH}_2\text{-}\\ \|\qquad\quad\ |\\ \text{O}\cdots\cdots\text{OH}\\ (-)\qquad(+)\end{array}$$

And such dipolorized molecules combine each other as follows.



$$\begin{array}{r}\text{-C-CH=C-CH}_2\text{-CH}_2\text{-}\\ \|\qquad\ \ |\qquad\qquad\quad\\ \text{O}\qquad\text{OH}\qquad\qquad\quad\\ \vdots\qquad\ \ \vdots\qquad\qquad\quad\\ \text{OH}\qquad\text{O}\qquad\qquad\quad\\ |\qquad\ \ \|\qquad\qquad\quad\\ \text{-CH}_2\text{-CH}_2\text{-C=CH-C-}\qquad\qquad\quad\end{array}$$

Anyhow, in this way the peroxide radical may be gradually decomposed inducing polymerization of fatty acid molecules.

This is also the reason why the sample, which lost its peroxide activity by standing or heating for longer time gained high viscosity.

SUMMARY

1. When ethyl ester of highly unsaturated fatty acid from cuttle fish oil was submitted to autoxidation in the presence of air, peroxide was formed. Its amount increased gradually, reached maximum and then decreased.

2. The toxicity of oxidized fatty acid ester ran parallel to the content of peroxide. Even the fatty acid, which once showed a high

toxicity, turned out to be non toxic when its peroxide contents were reduced by further heating or by long standing.

3. The rate of formation of peroxide and its amount were much influenced by temperature. At low temperature the rate was slow but the amount was large, while at higher temperature the rate was rapid, at the same time its decomposition was also quick, so that remaining peroxide content was much less.

4. It is of prime importance to examine the peroxide content in fish oil when this is applied for dietary use.

The author expresses his heartful thanks to Prof. Keizo Kodama for his continuous guidance through the course of this research, and at the same time, also wishes to thank Prof. S. Misaka and Dr. G. Shibamoto for their encouragement for this work and T. Yoshikawa for his assistance in carrying out the experiments.

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The Journal of Biochemistry, Vol. 41, No. 5, 1954

STUDIES ON THE ANTERIOR PITUITARY GONADOTROPHIN

III. CARBOHYDRATE CONTENTS OF BEEF INTERSTITAL CELL STIMULATING HORMONE (ICSH)

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(Received for publication, July 15, 1954)

The gonadotrophic hormone, whether it is derived from pituitary or chorionic tissue, belongs to glycoprotein and contains hexose and hexosamine.

Both human urinary gonadotrophin (HCG) and pregnant mare serum gonadotrophin (PMSG) contain galactose as their hexose component and the ratio of hexose to hexosamine is about 2:1. On the other hand, pituitary gonadotrophins, (FSH and ICSH) contain mannose and hexosamine in the proportion 1:1. Of course the carbohydrate content of pituitary interstitial cell stimulating hormone (ICSH) varies from pituitary follicle stimulating hormone (FSH) and also varies with species of animal from which it was extracted.

Sheep ICSH contains 4.5 per cent mannose and 5.8 per cent hexosamine (*1*) while swine ICSH contains 2.8 per cent mannose and 2.2 per cent hexosamine (*2*). It was thought, therefore, that carbohydrate analysis of beef ICSH might prove to be of considerable interest.

In the present paper of this series hexose and hexosamine contents were estimated with beef ICSH which was prepared by the method previously described (*3*).

Hexose was determined by the method of Sörensen and Haugaard (*4*), and modified by Stary *et al.* (*5*) Hexosamine was determined by the method of Elson and Morgan (*6*), and modified by Stary *et al.* (*5*)

EXPERIMENTAL

Beef ICSH—Beef ICSH was prepared as previously (*3*) by methanol-acetate extraction and rivanol precipitation.

Orcin Reagent—35 ml. of 3 per cent orcin solution was added to the mixture of

15 ml. conc. sulfuric acid and 35 ml. distilled water.

Acetylacetone Reagent—0.5 ml. of acetylacetone was dissolved in 25 ml. of 0.5 *N* sodium carbonate.

Aldehyde Reagent—0.4 *g.* of *p*-dimethylaminobenzaldehyde was dissolved in the mixture of 15 ml. ethanol and 15 ml. conc. hydrochloric acid.

Hexose Determination—1 ml. of the orcin reagent and 7.5 ml. of sulfuric acid (d=1.49) were added to 1 ml. of the hormone solution (containing about 0.1–0.3 mg. carbohydrate) and this mixed solution was heated at 80° for 20 minutes in a dark room. Then the solution was cooled in running tap water and added with 10 ml. of distilled water and estimated colorimetrically by using photoelectric colorimeter at 420 mμ and 520 mμ within 1 hour after reaction. Control experiment was carried out with mannose and galactose.

The results are shown in Table I and Fig. 1. The ratio of E_{420}/E_{520} was varied with the sort of carbohydrate and this ratio of beef ICSH was resembling that of mannose.

TABLE I

Comparison of Colours Produced by Hexoses in Orcinol Reaction

Sample		E_{520}	E_{420}/E_{520}
	mg.		
Mannose	0.10	0.138	6.54
〃	0.15	0.207	5.97
〃	0.20	0.285	5.34
〃	0.25	0.355	4.98
〃	0.30	0.418	4.78
Galactose	0.10	0.155	4.77
〃	0.15	0.230	4.54
〃	0.20	0.302	4.30
〃	0.25	0.355	4.25
〃	0.30	0.424	4.17

Hexose Contents of Beef ICSH

Sample		E_{520}	E_{420}/E_{520}	Mannose content	
Beef ICSH	*mg.*			*mg.*	%
G101	2.8	0.240	5.65	0.172	6.14
〃	5.7	0.440	4.48	0.322	5.65
〃	7.3	0.590	3.40	0.430	5.89
G100	2.6	0.225	5.68	0.160	6.15
〃	3.8	0.280	5.30	0.200	5.26
G102	3.5	0.267	5.48	0.190	5.64
〃	2.5	0.217	5.64	0.155	6.20
				Mean	5.85

Glucosamine Determination—9.6 mg. of beef ICSH was hydrolyzed with 7 ml. of

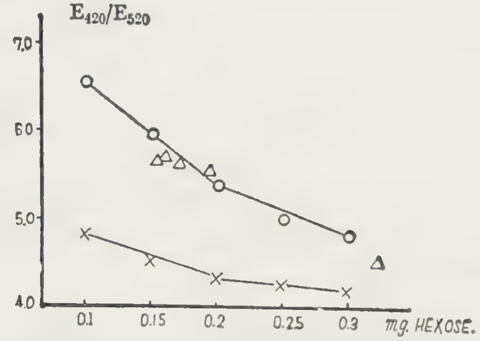

FIG. 1. E_{420}/E_{520} value of mannose, galactose and beef ICSH.
○ mannose : × galactose : △ beef ICSH.

4 *N* hydrochloric acid at 100° for 12 hours, neutrallized with sodium hydroxyde and made up to 10 ml. with distilled water.

Acetylacetone reagent (2 ml.) was added to 4 ml. of hydrolyzate and heated in a boiling water-bath for 20 minutes. After cooling this solution 5 ml. of 99 per cent ethanol and then 2 ml. of aldehyde reagent were added with vigorous shaking and allowed to stand for 45 minutes. 2 ml. of 99 per cent ethanol was added and estimated colorimetrically at 530 mμ. Control experiment was carried out with standard glucosamine solution.

The results are shown in Table II.

TABLE II

Hexosamine Contents of Beef ICSH

Sample		E_{530}	Glucosamine content	
			mg.	%
Glucosamine	0.162 mg.	0.200		
//	0.324	0.445		
//	0.486	0.610		
G101	3.84	0.287	0.23	5.98
G100	2.48	0.195	0.155	6.30
			Mean	6.14

DISCUSSION

The carbohydrate of beef ICSH was studied qualitatively with paper chromatography and paper electrophoresis and determined as mannose.

According to the orcin method, the ratio of E_{420}/E_{520} also showed that the hexose component of beef ICSH is mannose.

As shown in the Table, beef ICSH contains 5.85 per cent of mannose and 6.14 per cent of glucosamine; this value is varied from that of sheep or swine but the ratio of mannose to glucosamine is likewise *ca.* 1 : 1.

The author wishes to express his gratitude to Dr. K. Takeda for his kind guidance and encouragements through this work and to Mr. Y. Noda for his much technical asistance.

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The Journal of Biochemistry, Vol. 41, No. 5, 1954

STUDIES ON PTERIDINES

VI. THE PTERIN FROM BOMBYX MORI (SILKWORM)[+] AND RANA NIGROMACULATA (FROG)[++]

By SABURO NAWA,* MIKIYASU GOTO,** SADAO MATSUURA,**
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(Received for publication, July 16, 1954)

We have previously isolated a pteridine from the eggs of *Bombyx mori*, which was tentatively named leucopterin-B (*1*). We have subsequently succeeded in obtaining this compound in a crystalline form (*2*) and have found that, similar to the pteridine obtained from the scales of cap, it belonged to the isoxanthopterin series. Since oxidation with alkaline $KMnO_4$ yielded a small amount of isoxanthopterin-6-carboxylic acid (V) (identified by paper chromatography), we had assumed that leucopterin-B was an isoxanthopterin derivative with a 6-side chain (*3*). However, further studies have shown the formation of (V) to be due to the contamination of an impurity, and the data provide evidence for the identity of leucopterin-B with isoxanthopterin itself.

A purple-fluorescent substance has also been extracted from the skins of *Rana nigromaculata*; though we have not succeeded in obtaining the substance in a crystalline condition, the facts mentioned in this paper indicate that this is also identical with isoxanthopterin. Hama has previously proposed the names of Rana-chrome 1, 2, 3, 4, 4B,5 and 6 for the fluorescent substances from *Rana nigromaculata*, the respective substances being named in decreasing order of Rf values ($BuOH:AcOH:H_2O=4:1:1$) (*4*); the present substance corresponds to rana-chrome 4. In the course of work on the purification of this compound, we obtained a fairly large amount of a crystalline substance, which has been identified as guanine by UV spectra, analytical data and qualitative tests.

[+] Presented at the 5th annual meeting of the Chemical Society of Japan, April 1952, and [++]at the 26th annual meeting of the Japanese Biochemical Society, April 1954.

EXPERIMENTAL RESULTS AND DISCUSSION

7-Hydroxy pterins do not have a characteristic melting point and decompose above 300°. They are soluble in alkali but are almost insoluble in neutral or acidic media and common organic solvents. The presence of the pterin may be detected in an extremely dilute aqueous solution on account of the strong fluorescence; the aqueous solutions of leucopterin-B and rana-chrome 4 possess a strong purple fluorescence. The procedures used for the extraction of both substances were essentially the same as those descirbed in the previous paper (*2*) in this series. The UV spectra of leucopterin-B and isoxanthopterin in 0.1 *N* NaOH were superimposable, and the UV spectrum of rana-chrome 4 was practically identical with isoxanthopterin in 1 *N* NaOH (Table I).

TABLE I

Compound	Solvent	max. (mμ)	(log ε)	max. (mμ)	(log ε)	min. (mμ)	(log ε)
Leucopterin-B:	0.1 *N* NaOH	255	4.07	340	4.16	290	3.45
Isoxanthopterin:	〃	255	4.09	340	4.19	290	3.48
Rana-chrome 4:*	1 *N* NaOH	255	3.91	339	3.96	290	3.33
Isoxanthopterin:	〃	255	3.93	340	4.02	290	3.29
Analysis:	Calcd. for $C_6H_5O_2N_5$:	C, 40.22;	H, 2.80;	N, 39.11			
	Found. (Leucopterin-B):	C, 40.77;	H, 2.96;	N, 39.24			

* The slight inconsistency is presumably due to the fact that the substance is non-crystalline.

The various synthetic 7-hydroxy pterins were closely related in many respects (*e.g.*, solubility, fluorescence, UV spectra) (*5*) and paper chromatography provided the simplest means for characterization (Table II). The compounds were submitted to several chemical reactions which only attacked the side chain, and the Rf values of respective compounds before and after the reactions are also listed in the Table II.

Thermal Decomposition—When the dried powder was heated for one hour at 260° *in vacuo*, (V), (VI,) (VII) and (VIII) yielded (I), (II), (III) and (IV), respectively. The β-carboxylic group in β-(isoxanthopteryl-6)-carboxylic acid (IX) remained unchanged.

MnO_2 Oxidation—A small amount (enough for paper chromatography) of the pterin was dissolved in 5 per cent NaOH in a test tube and after addition of pulverized MnO_2, the mixture was stirred and heated on the bath for 10 minutes, when the side chains of compounds (VI),

TABLE II

The Rf Values of Respective Compounds before and after the Reactions

No.	Compound				Heating at 260°		MnO_2 oxidation		Al-Hg treatment		Esterification (methyl)
	Side chain	a*	b*	c*	a	b	a	b	a	b	a
	Leucopterin-B	0.25	0.32	0.52	No change		No change		No change		No change
	Rana-chrome 4	0.25	0.32	0.52	//		//		//		//
I	$-H$	0.25	0.32	0.52	//		//		//		//
II	$-CH_3$	0.32	0.30		//		//		//		//
III	$-C_2H_5$	0.54	0.35		//		//		//		//
IV	$-C_3H_7$	0.70	0.39		//		//		//		//
V	$-COOH$	0.08	0.57		0.25 (I)	0.32	//		0.25 (I)	0.32	//
VI	$-CH_2COOH$	0.22	0.61		0.32 (II)	0.30	0.08 (V)	0.57	No change		0.45
VII	$-CH(CH_3)COOH$	0.35	0.65		0.54 (III)	0.35	0.08 (V)	0.57	//		0.77
VIII	$-CH(C_2H_5)COOH$	0.61	0.67		0.70 (II)	0.39	0.08 (V)	0.57	//		0.85
IX	$-CH_2CH_2COOH$	0.25	0.48		No change		No change		//		0.80
X	$CH(OH)CH_3$	0.35	0.26		. . .		0.08 (V)	0.57	0.25 (I)	0.32	. . .
XI	$-CH_2CH(OH)CH_3$	0.42	0.47		. . .		No chnge		No change		. . .
XII	$-CH_2NH_2$	0.07	0.30		. . .		0.08 (V)	0.57	0.25 (I)	0.32	. . .
XIII	$-CH_2COCH_3$	0.40	0.47		. . .		0.08 (V)	0.57	0.25 (I)	0.32	. . .

* Solvent: a, Butanol-acetic acid-water (4 : 1 : 1); b, 4% Na-citrate solution; c, Lutidine-collidine-water (1 : 1 : 1)

(VII), (VIII), (X), (XII) and (XIII) were all oxidized to give R= –C-OOH (V). The above mentioned oxidation by alkaline $KMnO_4$ not only oxidized all of the side chains to a carboxylic group but also attacked the nucleus in most cases to give unidentified and unsatisfactory results, whereas the MnO_2 oxidation only took place with certain side chains and left the pteridyl nucleus intact.

Al-Hg Reduction (*6*)—(V), (X), (XII) and (XIII) were cleaved from the α-C atom to give isoxanthopterin (I).

Esterification—When suspended in absolute methanol (or ethanol) and saturated with dry HCl gas, the pterins possessing carboxylic side chains, (VI), (VII), (VIII) and (IX), dissolved and afforded their esters. Isoxanthopteryl carboxylic acid was an exception and not esterified under the mentioned conditions.

As shown in the Table II, no inconsistency of leucopterin-B and rana-chrome 4 with isoxanthopterin could be obserued in respect to the various reactions. The UV spectra of the two substances and the analytical data of leucopterin-B also agreed with isoxanthopterin. The inertness towards all of the reactions suggested the absence of a functional group, and the unidentity with compounds with simple alkyl side chains was apparent from paper chromatography.

Hence, presumably leucopterin-B and rana-chrome 4 are identical with isoxanthopterin.

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RECHERCHES SUR LA FORMOL-GÉLIFICATION DES PROTÉINES

II. INFLUENCE DES SELS SUR LA COURBE DE FORMOL-GÉLIFICATION DE L'EUGLOBULINE*

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Il y a trois ans (*1–3*), nous avons étudié de quelle manière les fractions protidiques du sérum réagissent en présence du formol, en suivant la variation de leur viscosité à divers pH. Ce faisant nous avons constaté que, parmi ces fractions, seule l'euglobuline réagit d'une manière sensible au formol. Le phénomène de formol-gel (réaction de Napier) est caractéristique de l'euglobuline dont la viscosité maximum apparaît entre pH 6 et pH 9, et plus particulièrement entre pH 7 et pH 8.

Ensuite de ces recherches nous avons suivi dans notre communication précédente avec l'euglobuline qui est fort réactive au formol, et l'albumine qu'on croyait peu réactive à lui, comment elles réagissent et se transforment si on change le degré de concentration du formol à y ajouter. Et nous avons constaté que la courbe de viscosité de l'euglobuline (γ-globuline**) qui atteint le point le plus élevé entre pH 7 et pH 8 dans les conditions normales de la formol-gélification s'élève encore plus si le formol est plus concentré, mais elle s'abaisse au contraire et finalement disparaît complètement, si l'on accroit la concentration du formol. En même temps on remarque que le pH oú se trouve le sommet de la courbe de viscosité, se déplace du côté de l'acidité. Quand le formol est très concentré, on peut observer apparaître un nouveau sommet de la courbe de viscosité aux environs de pH 5. L'euglobuline et l'albumine présentent toutes deux ce phénomèns. Si l'on prend en considération les résultats de l'expérience réalisée par Tanda (*6*), qui montrent que l'euglobuline dénaturée par un alcali fort augmente sa viscosité ou se coagule aux abords de pH 5, et d'autre part ceux de l'expérience effectuée par Sano (*7*), qui prouvent que le sérum exposé à une chaleur modérée augmente sa viscosité vers pH 5, aprés addition de formol, il nous semble que l'augmentation de la viscosité auprès de pH 5 est un phénomène causé par la dénaturation de la protéine.

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* Le sommaire de ce mémoire a été présenté au 23e Congrès de Biochimie du Japon, Avril 1951 (*1*).

** L'euglobuline, que nous avons employée, contenait à peu prés 85 p. 100 de γ-globuline, le reste étant composé de fractions diverses principalement de β-globuline (voir la communication précédente (*4, 5*).)

Dans la présente communication nous suivrons l'influence des sels sur le phénoméne d'augmentation de la viscosité entre pH 7 et pH 8, lequel se tient étroitement lié à la formol-gélification (réaction de Napier). L'influence des sels sur les réactions de la solution de protéine, ou sur celles du sérum n'est pas encore bien établie. En particulier nous ne pouvons pas trouver d'études concernant l'influence des sels sur la formol-gélification. Il y a bien certes quelques savants qui ont effectué leurs expériences en employant des solutions tampon, mais sans considérer cette influence.* Nous croyons que leurs résultats d'expérience ainsi obtenus doivent être examinés avec prudence, parce qu'il nous faut considérer l'influence des sels qui composent la solution tampon. Pour cette raison nous avons suivi la méthode que Suzuki (*9–14*) avait déjà appliquée au sérum de Kala-azar afin d'etudier la formolgélification des fractions protidiques du sérum en suivant la variation de leur viscosité a divers pH. Nous nous sommes servis de ClH et de OHNa dissous dans une solution de ClNa à 0,9 p. 100, en utilisant l'action tampon de la solution de protéine. Par ce procédé nous avons préparé, à aux pH différents une série de solutions de protéine de quantité égale et du même degré de concentration. Il va sans dire qu'ainsi l'action tampon de la solution de protéine diffère dans chaque cas. Le pH, enregistré en ajoutant la même quantité de ClH ou de OHNa, n'était pas toujours le même. Mais, mesurant la viscosité de chaque solution, nous avons comparé graphiquement ses variations en rapport avec pH, autrement dit nous avons traité la viscosité comme une fonction de pH, dans lequel la protéine réagit à l'action du formol. De la sorte pH continuait de varier du fait de l'action tampon de la solution de protéine, mais ces variations ne présentaient plus d'importance. Il suffisait de mesurer pH avec précision. Malgré l'apparence compliquée de cette méthode, elle est, en pratique très simple et sûre.

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* Par exemple, Chopra et Choudhury (*8*) qui avaient préalablement fixé à divers pH du sérum de Kala-azar, en ajoutant une solution tampon, ont montré que la formol-gélification se produit en un laps de temps minimum, à pH 6,94 ou à à pH 7,27.

En recourant à cette méthode nous avons effectué diverses expériences dont nous avons donné le compte-rendu dans les deux communications précédentes et considéré à cette occasion l'influence des sels. Dans ce mémoire nous verrons l'influence des sels sur la formol-gélification. Pour cela nous avons choisi respectivement ClNa, SO_4Na_2 et Cl_2Ca, comme sels de types I–I, II–I et I–II.

MÉTHODE D'EXPÉRIENCE

Recourant à la méthode décrite précédemment (lère communication) (*4*, *5*), nous avons préparé à divers pH des solutions à concentration égale d'euglobuline en utilisant ClH et OHNa dissous dans une solution de ClNa à 0,9 p. 100. Les expériences que nous relatons ici, ont été réalisées en ajoutant une goutte de formol à 1 ml. de la solution de protéine. (La concentration finale du formol était à peu près de 1 p. 100).

RÉSULTATS D'EXPÉRIENCE

I. Influence de ClCa (un des Sels de Type I–I) sur la Courbe de Viscosité de l'Euglobuline.

Tout d'abord, nous avons choisi ClNa comme sel de type I–I et observé l'influence de ce sel sur la courbe de viscosité de l'euglobuline. Nous avons obtenu les résultats donnés en graphiques ci-joints (Fig. 1, A). Pour suivre l'influence du sel, nous avons effectué des expériences avec une concentration très diluée d'euglouline. L'augmentation de la viscosité entre pH 7 et pH 8 n'a donc pas été très sensible, lorsque nous avons ajouté du formol à la solution de protéine. Nous avons obtenu deux courbes. En comparant la première représentant la viscosité après addition de formol et la seconde représentant la viscosité après addition de formol+ClNa, nous avons constaté que la première se trouvait un peu plus élevée que la seconde, du coté de l'alcalinité aux environs de pH 5. Cependant, en comparant les courbes décrites par des autres sels (type I–II et II–I), nous avons remarqué une élévation très sensible de la viscosité à concentration égale (mesurée en grammes-équivalants de ClNa, et nous en avons déduit que ClNa n'influence pas nettement sur la courbe de viscosité de l'euglobuline dans des limites assez larges de pH.

II. Influence de SO_4Na_2 (Un des Sels de Type II–I) sur la Courbe de Viscosité de l'Euglobuline.

Comme nous venons de le voir, la courbe de viscosité de l'euglobuline n'a pas montré de changements très sensibles après addition de ClNa. Par addition de SO_4Na_2 nous avons, au contraire, trouvé une forte augmentation de la viscosité entre pH 7 et pH 8 (dans les limites de la formol-gélification). Cependant ce qui a surtout attiré notre attention, c'est une convexité de la courbe de viscosité entre pH 3 et pH 4. Nous

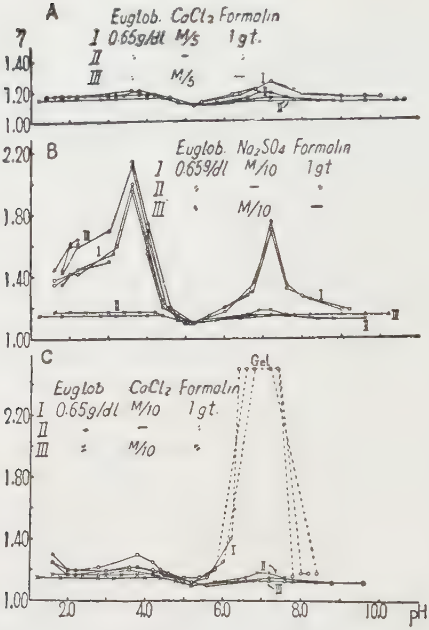

FIG. 1. Influence de NaCl (A), Na_2SO_4 (B) et $CaCl_2$ (C) sur la courbe de viscosité de l'euglobuline.

n'avons observé cette convexite qu'en cas d'addition de SO_4Na_2 et par conséquent nous pensons qu'il y a lieu de considérer que ce phénomène n'a aucun rapport avec la formol-gélification.

Ensuite nous avons étudié les rapports entre la concentration de SO_4Na_2 et la courbe de viscosité et nous avons obtenu les résultats donnés en graphique 2, qui montrent que l'élévation de la courbe de viscosité est fonction de la concentration du sel. En dehors de cela, rien de particulier à signaler. Nous montrons graphiquement (Fig. 3) la relation existant entre la concentration du sel et la viscosité entre pH 7 et pH 8 (les limites de la formol-gélification). Ce qui permet de constater qu'à mesure qu'augmente la concentration du sel, la viscosité s'accroit légèrement d'abord, mais de plus remarquablement. D'autre part la Fig. 3, B indique la relation entre la concentration du sel et la viscosité entre pH 3 et pH 4. Cette augmentation de la viscosité, causée par addition du sel, n'a évidemment aucun rapport avec la formol-gélification.

Par ailleurs nous avons observé deux convexités de la courbe de viscosité indiquant la formol-gélification vers pH 7–8 et pH 5, en fonction du temps (voir ; $1^{\text{ère}}$ communication). Dans ce cas là nous avons noté une relation à fonction parabolique entre pH 7 et pH 8, et une relation à fonction exponentielle aux abords de pH 5, entre la

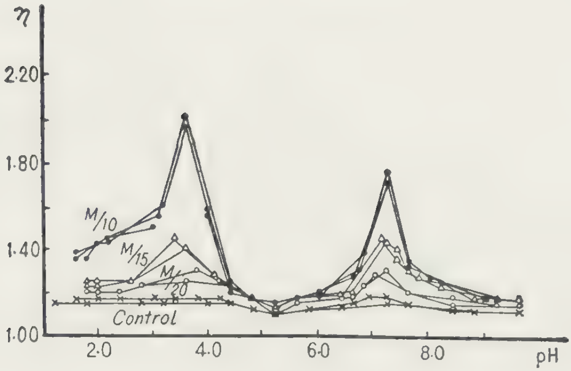

FIG. 2. Influence de Na_2SO_4 à divers degrés de concentration sur la courbe de formol-gélification de l'euglobuline.

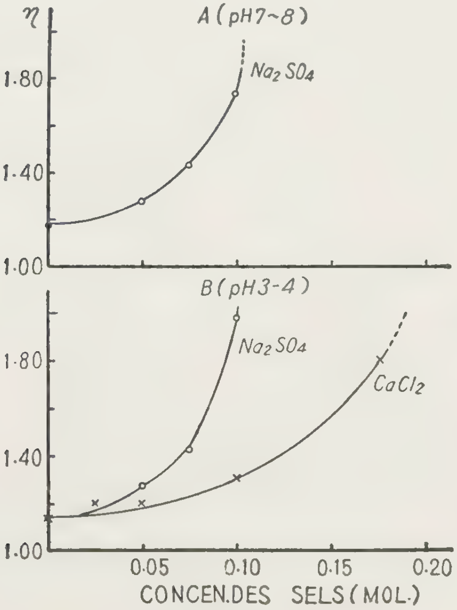

FIG. 3. Rapport entre les maximums de viscosité vers pH 7–pH 8 d'une part et vers pH 3–pH 4 de l'autre et la concentration des sels.

viscosité et le temps. Nous traitons des phénomènes en détail plus bas.

III. Influence de Cl_2Ca (Un des Sels de Type I–II) sur la Courbe de Viscosité de l'Euglobuline.

Nous avons choisi Cl_2Ca comme sel représentatif de type II–I et observé son influence sur la courbe de formol-gélification de l'euglobuline. La Fig. 1, C, montre les résultats de nos expériences qui se traduisent par deux courbes, la première correspondant à l'addition de formol seul et la seconde à une addition de formol et de Cl_2Ca. La première accuse une faible convexité. Ce phénomène est causé par la faible concentration de l'euglobuline. La seconde au contraire laisse apparaitre une élévation de la viscosité entre pH 7 et pH 8. Ensuite nous avons effectué plusieurs séries d'expéri ences similaires, chaque série à un degré différent (de $M/5$ à $M/40$) de concentration de Cl_2Ca. Dans toutes ces séries d'expériences, la gélification s'est produite entre pH 7 et pH 8 (Fig. 4). En considérant ces résultats, nous avons constaté que Ca^{++} est un facteur très efficace pour accélérer la formol-gélification. Nous avons en effet observé une convexité de la courbe de viscosité entre pH 3 et pH 4 en rapport avec l'accroissement de la concentration de Cl_2Ca. Ce phénomène apparait, que l'on ajoute ou non du formol, et également on l'a vu plus haut en cas d'addition de SO_4Na_2. Il semble donc qu'on doive le considérer comme un cas de gélification dû à l'addition d'un sel. Cependant l'élévation de la courbe de viscosité entre pH 3 et pH 4 par addition de SO_4Na_2 à concentration égale (mesurée en grammes-équivalents), et quand on augmente la concentration de Cl_2Ca, la viscosité augmente rapidement (voir graphique 3, B).

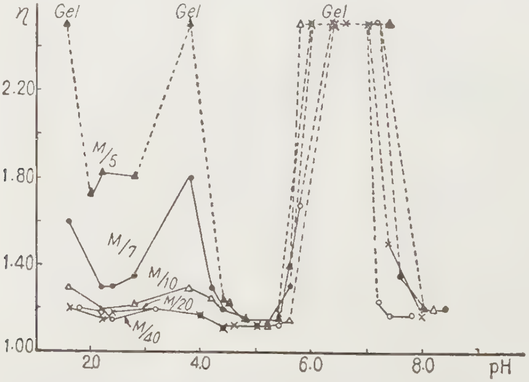

FIG. 4. Influence de Cl_2Ca à divers degrés de concentration sur la courbe de formol-gélification de l'euglobuline.

DISCUSSION

Ainsi, nous avons observé l'influence des sels de types I–I, I–II et

II–I sur la courbe de viscosité de l'euglobuline, pour constater que cette influence se manifeste vers pH 7–pH 8 et pH 3–pH 4. Cependant le phénomène d'augmentation de la viscosité entre pH 3 et pH 4 n'a aucun rapport avec la formol-gélification.

Nous devons considérer donc ces deux cas séparément. Tout d'abord nous savons que les pH 7–pH 8 constituent les limites de la formol-gélification, et que l'augmentation de la viscosité n'est possible que par addition du formol. Autrement dit l'addition des sels ne fait que relever la courbe de viscosité dûe à l'action du formol. Au contraire nous avons observé une augmentation de la viscosité entre pH 3 et pH 4 seulement par addition des sels à la solution d'euglobuline et pas par addition du formol. Par conséquent, il semble qu'on doive regarder cette élévation de la viscosité comme un cas de gélification causée du sel, le moindre rapport avec la formol-gélification. Par ailleurs, Suzuki (*13–14*) a noté une convexité de la courbe de viscosité entre pH 3 et pH 4, par addition d'acide citrique, etc., et le mécanisme de ce phénomène reste à éclaircir. Cependant, étant donné les limites de pH, il semble présenter certaines affinités avec le cas en question (augmentation de la viscosité entre pH 3 et pH 4 par addition d'un sel). De plus, entre pH 3 et pH 4 et également entre pH 7 et pH 8, chaque sel exerce une influence particulière. En ajoutant ClNa (un cas des sels de type I–I), nous n'avons pas enregistré d'élévation de la courbe de viscosité entre pH 3 et pH 4 et nous n'avons pas non plus observé d'accroissment sensible de la formol-gélification entre pH 7 et pH 8. Au contraire, en ajoutant SO_4Na_2 (un des sels de type II–I) ou Cl_2Ca (un des sels de type I–II), nous avons noté une augmentation de la viscosité dans ces deux limites de pH (pH 3–pH 4 et pH 7–pH 8). L'élévation de la courbe de viscosité entre pH 7 et pH 8 (limites de la formol-gélification) par addition de Cl_2Ca a cependant été plus notable que celle provoquée par addition de SO_4 Na_2. En ajoutant Cl_2Ca nous avons pu observer la gélification de la solution de protéine, lorsque la concentration du sel était très faible (*M*/40 par exemple). Au contraire, l'élévation de la courbe de viscosité entre pH 3-pH 4 par addition de So_4Na_2 était plus considérable que celle provoquée par l'action de Cl_2Ca. Cette élévation a lieu dans les deux cas avec ou sans addition de formol. Si l'on examine ces phénomènes ci-dessus en considérant le point iso-électrique, on constate immédiatement que l'action de Cl_2Ca est très forte du côté de l'alcalinité où la protéine réagit comme anion. Par ailleurs, l'action de SO_2^{--} est plus forte que celle de Cl_2Ca du côté de l'acidité où la protéine réagit comme cation. Cependant la réaction de combinaison entre la protéine et les sels n'est pas aussi simple qu'on pourrait le supposer d'après ce qui précède. La poursuite de nos recherches apporte peu à peu des faits nouveaux en la matière et nous pensons que nos travaux à venir éclairciront ce problème.

RÉSUMÉ

1. Nous avons observé l'influence des sels sur la courbe de viscosité de l'euglobuline dans certaines limites assez larges de pH.

2. Nous avons constaté que l'addition de ClNa (un des sels de type I–I) n'a qu'une faible influence dans des limites assez larges de pH.

3. En ajoutant SO_4Na_2 (un des sels de type II–I), nous avons noté un accroissement de la formol-gélification entre pH 7 et pH 8. D'autre part une convexité de la courbe de viscosité entre pH 3 et pH 4 apparaissait. Ce dernier phénomène a été observé en ajoutant ou non du formol. Par conséquent il n'a aucun rapport avec la formol-gelification.

4. En ajoutant Cl_2Ca (un des sels de type I–II), nous avons observé une sensible élévation de la courbe de viscosité, dans les limites de la formol-gélification (entre pH 7 et pH 8). Cependant l'augmentation de la viscosité entre pH 3 et pH 4 exclusivement par addition de Cl_2Ca (sans rapport avec la formol-gélification) s'est révélée plus faible que celle dûe à l'action de SO_4Na_2.

Nous exprimons notre gratitude au Docteur Araya, Professeur à la Faculté Dentaire, Docteur Anan, Professeur adjoint à la Faculté de Médecine, Docteur Kobayashi, Professeur adjoint à la Faculté Dentaire, pour des conseils qu'ils ont portés à ce travail et aussi à Monsieur J. Renard qui a eu la bonne volonté de corriger des manuscripts en Francais.

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